Identification of new drug targets in adrenocortical carcinoma through targeted mRNA analysis

Identifikation neuer Drug Targets im Nebennierenrindenkarzinom durch gezielte mRNA-Analyse

Doctoral thesis for a medical doctoral degree
at the Graduate School of Life Sciences
Julius-Maximilians-Universität Würzburg
Section Biomedicine

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Würzburg 2020

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1. Introduction

1.1. Adrenal gland

The adrenal gland is a paired organ located primary retroperitoneal at the cranial part of both kidneys and weights approximately 4-5 g. It is one of the main sites of hormone production in the human body and is divided in two main structures while being encapsulated in connective tissue: the outer cortex producing steroid hormones and the medulla in the center producing catecholamines. Consisting of three zones the cortex can be further histologically and functionally differentiated. While the outermost zone, zona glomerulosa, is characterized by ovoid cell clusters and responsible for the production of mineralocorticoids like aldosterone, the second and thickest layer, the zona fasciculata, consists of cells arranged in parallel cords and synthesizes glucocorticoids like cortisol whereas the zona reticularis as the inner most layer of the cortex with a net-like cell formation produces mainly adrenal androgens like DHEA (dehydroepiandrosterone).

1.2. Adrenocortical carcinoma

1.2.1. Epidemiology

ACC (adrenocortical carcinoma) is a rare malignant tumor originating from the cortex of the adrenal gland with a heterogeneous but generally unfavorable prognosis. The incidence is at approximately 1-2 per million (Cutler et al., 1975, Kebebew et al., 2006) with a female preponderance at a ratio of female to male at about 1.5:1 (Luton et al., 1990, Wooten and King, 1993, Icard et al., 2001, Allolio and Fassnacht, 2006). A bimodal age distribution with peaks in childhood and the fourth to fifth decades was observed (Wajchenberg et al., 2000). While germline mutations of tumor suppressor gene *TP53* (tumor protein p53) were reported in 50-80% of pediatric ACC, an incidence up to 15 fold was observed in children from southern Brazil compared to children worldwide due to a specific germline mutation in the *TP53* gene (Ribeiro et al., 2001, Custodio et al., 2012).

1.2.2. Pathogenesis

Studies of familial diseases revealed genetic predisposition in several cases, most commonly inactivating germline *TP53* mutations associated with the Li-Fraumeni syndrome (Ribeiro et al., 2001, Herrmann et al., 2012, Raymond et al., 2013a). In rarer

cases, alterations of the imprinted 11p15 region implicated with the Beckwith-Wiedemann syndrome (Henry et al., 1989, Libe et al., 2007) as well as mutations of the *MEN1* (menin 1) gene associated with MEN1 syndrome (multiple endocrine neoplasia type 1) (Schulte et al., 2000, Langer et al., 2002, Gatta-Cherifi et al., 2012) and mutations in genes involved in DNA mismatch repair associated with Lynch syndrome (Karamurzin et al., 2012, Broaddus et al., 2004, Raymond et al., 2013b) were observed in ACC. Moreover, ACC have been reported in several cases of patients with FAP (familial adenomatous polyposis) affecting *APC* (adenomatous polyposis coli) (Wakatsuki et al., 1998, Painter and Jagelman, 1985), neurofibromatosis type 1 affecting *NF1* (neurofibromin 1) (Wagner et al., 2005) and Carney complex affecting *PRKAR1A* (protein kinase CAMP-dependent type I regulatory subunit alpha) genes (Anselmo et al., 2012, Morin et al., 2012) (Else Mol Cell Endocrinol 2013).

At tumor level, overexpression of the gene *IGF2* encoding for the insulin-like growth factor 2 occurs in about 90% of ACC mainly due to paternal unidisomy at the 11p15 locus in sporadic ACC (Gicquel et al., 2001, Giordano et al., 2003). Moreover, somatic mutations in gene *CTNNB1* (catenin beta 1) were reported in both ACC and ACA (adrenocortical adenoma), leading to abnormal activation of the Wnt/β-catenin pathway (Gaujoux et al., 2011). Recently two large pan-genomic characterization studies were conducted on ACC, describing additional mutations in *ZNFR3* (zinc and ring finger 3)*, DAXX* (death domain associated protein)*, MED12* (mediator complex subunit 12)*, TERT* (telomerase reverse transcriptase) and *PRKAR1A* among others (Assie et al., 2014, Zheng et al., 2016). Furthermore, alteration in several microRNAs (Tombol et al., 2009, Caramuta et al., 2013) as well as in the methylation profile (Fonseca et al., 2012, Assie et al., 2019) have been proposed to contribute to pathogenesis of ACC.

1.2.3. Clinical presentation

Approximately 60% of ACC exhibit steroid hypersecretion responsible for specific clinical syndromes, most commonly rapidly developing Cushing's syndrome with or without virilization by hypercortisolism and/or androgen excess. An excess of mineralocorticoids and estrogens occur rather rarely. Absence of clinical symptoms in spite of increased hormonal activity can be due to predominant secretion of steroid precursors or impediment of intratumoral steroidogenesis. About 30-40% of patients

suffer from non-specific symptoms from an abdominal mass such as nausea, vomiting and abdominal or dorsal pain, while 10-15% of tumors are incidentalomas, incidentally discovered by imaging for other purposes. Systemic B symptoms as fever, night sweats and weight loss occur less frequently (Arlt et al., 2011, Kerkhofs et al., 2015, Fassnacht et al., 2013, Fassnacht et al., 2018).

1.2.4. Diagnostic work-up

1.2.4.1. Endocrine work-up

As the majority of ACC show autonomous hormone secretion, endocrine work-up serves to prove the adrenocortical origin and the dignity of the tumor. Hormone secretion patterns may also be used as tumor marker to detect recurrences as well as to evaluate prognosis of ACC patients (Fassnacht et al., 2004, Libe et al., 2007, Fassnacht et al., 2013). According to the guidelines of the ESE (European Society of Endocrinology) and recommendation of the ENSAT (European Network for the Study of Adrenal Tumors), hormonal work-up should include basal ACTH (adrenocorticotropin), DHEA-S (dehydroepiandrosterone sulfate), 17-OH-progesterone, androstenedione, testosterone in women, 17-beta-estradiol in men and postmenopausal women, 11-deoxycortisol and potassium. A dexamethasone suppression test and free cortisol in 24-h urine is used to detect hypercortisolism as autonomous cortisol secretion would make postoperative glucocorticoid treatment necessary to avoid adrenal insufficiency.

Fractionated metanephrines in 24-h urine or free plasma-metanephrines is measured to exclude pheochromocytoma. Additionally, the aldosterone/renin ratio is determined in patients with hypertension or hypokalemia. Urine steroid metabolite profiling by gas chromatography-mass spectrometry can further help to determine the dignity of adrenal tumors in cases with indistinct standard diagnostic work-up (Arlt et al., 2011, Bancos et al., 2020), but is not yet established for routine use.

1.2.4.2. Imaging

Imaging of abdomen and pelvis by CT (computerized tomography) scan or MRI (magnetic resonance imaging) is recommended in addition to a chest CT for the presence of metastatic disease as well as bone or brain imaging in case of suspicion for metastases. Generally, differentiation between ACA and ACC deploys several criteria as appearance

and size as well as the lipid content expressed in density in unenhanced CT (low lipid content with > 10 HU (Hounsfield unit) for ACC vs high lipid content with ≤ 10 HU for ACA) (Fassnacht et al., 2016, Dinnes et al., 2016). As, however, approximately a third of adrenal adenomas are known to be lipid-poor, contrast-enhanced CT may help to ascertain diagnosis in ambiguous cases as rapid contrast media washout after 10-15 min (cutoff 60%) suggest a benign adrenal tumor. MRI can additionally contribute to characterize an adrenal tumor as ACC appear isointense to hypointense on T1-weighted images, hyperintense on T2-weighted images and show a loss of signal on chemical shift imaging (Bharwani et al., 2011). Functional imaging by FDG-PET (fluorodeoxyglucose-positron emission tomography) show high 18F-fluorodeoxyglucose uptake in malignant adrenal lesions. It has, in particular, high sensitivity and negative predictive value for adrenal lesions and is yet not established for routine use in ACC patients (Groussin et al., 2009). [^{11}C]MTO (metomidate) and [^{123}I]IMTO (iodometomidate), analogs of etomidate, inhibit steroid synthesis by binding to adrenocortical CYP11B (cytochrome P450 11B) enzymes and were investigated in a small cohort for the use as adrenocortical-specific tracer for scintigraphy in order to differ between ACC and non-adrenocortical metastases (Hahner et al., 2008, Hennings et al., 2006, Kreissl et al., 2013).

1.2.4.3. Pathology

Only the pathological examination of the primary tumor allows the confirmation of the diagnosis of ACC. Macroscopically, ACC appear as large inhomogeneous masses, often with hemorrhage or necrosis and indications of tumoral invasion. Microscopically, adrenocortical origin of the tumor can be verified by HE (hematoxylin and eosin) staining and additionally ascertained by SF1 (splicing factor 1) protein expression in indistinct cases (Sbiera et al., 2010). Characterization of adrenal tumors is mainly facilitated by the Weiss system, a score including nine criteria concerning architecture, nucleus and presence of invasion (Weiss, 1984, Sasano et al., 2006). A Weiss score of ≥ 3 defines an ACC, while a score < 2 defines an adrenal adenoma. A score of 2-3 may refer to a borderline tumor. However, correct diagnosis may be challenging due to inter-observer variabilities and a review by an expert adrenal pathologist is suggested in unclear cases as misdiagnoses occur in 9-13% (Johanssen et al., 2010, Duregon et al., 2015).

Additionally, the proliferation marker Ki67 can help to characterize the aggressiveness of the tumor and was also shown to be useful as a prognostic biomarker (Beuschlein et al., 2015, Miller et al., 2010).

1.2.5. Staging

Cancer staging describes the growth and spread of the tumor and is generally the most important prognostic factor. Herein, based on the Lee classification from 1995, the ENSAT staging system for ACC provides a superior distinction and concordance with prognosis in comparison to other proposed classification systems (Fassnacht et al., 2009). Generally, stage I and II tumors are strictly localized while stage III is defined by infiltration into surrounding tissue, positive regional lymph nodes or a tumor thrombus in the vena cava or the renal vein. Presence of distant metastases mark stage IV tumors. The detailed classification according to the ENSAT staging system is listed in table 1.1.

Table 1.1: ENSAT classification system (Fassnacht et al., 2009).

ENSAT tumor stage	TNM
I	T1 N0 M0
II	T2 N0 M0
III	T3-4 N1 M0
IV	any T any N M1

Abbreviations: ENSAT = European network for the study of adrenal tumors; T1 = tumor ≤ 5 cm; T2 = tumor > 5 cm; T3 = tumor infiltration into surrounding tissue; T4 = tumor invasion into adjacent organs or venous tumor thrombus in vena cava or renal vein; N0 = no positive lymph nodes; N1 = positive lymph node(s); M0 = no distant metastases; M1 = presence of distant metastasis.

1.2.6. Therapy

1.2.6.1. Surgery

Complete tumor resection is currently the only curative treatment option and is mainly applied to localized tumors (Grubbs et al., 2010) but is also considered for patients with tumor recurrence and a disease-free interval of at least 12 months. On the other side, debulking surgery in ACC patients with widespread metastatic disease is only recommended in a palliative setting (i.e. for patients with overt Cushing syndrome) or for

selected patients with favorable characteristics (Schteingart et al., 2005, Kerkhofs et al., 2013, Erdogan et al., 2013, Ronchi et al., 2014a).

Despite being investigated by several studies, the decision between an open adrenalectomy and laparoscopic adrenalectomy remains controversial. Tumor size and presence of invasion are essential for the choice of surgical approach and it is recommended that only tumors with a diameter < 6 cm with no evidence of local invasion are treated by laparoscopic procedures (Jurowich et al., 2013, Fassnacht et al., 2018). With regard to the extent of resection, locoregional lymph node dissection might be beneficial for diagnostics and clinical outcome (Reibetanz et al., 2012) and while systematic nephrectomy in the absence of gross local invasion is not required, advanced local tumors with infiltration to nearby structures should be resected *en bloc* to achieve margin-free complete resection (Gaujoux and Brennan, 2012, Mihai et al., 2012). Furthermore, surgery performed by an experienced surgeon can also positively influence the outcome (Kerkhofs et al., 2013, Lombardi et al., 2012).

Currently, the relapse rate for ACC patients undergone radical resection is estimated at 30-50% with median disease-free survival being 12 months (Gonzalez et al., 2007, Fassnacht et al., 2010, Ronchi et al., 2014a).

1.2.6.2. Mitotane

Currently, the adrenolytic drug mitotane (1,1-dichlor-2-(2-chlorphenyl)-2-(4-chlorphenyl)-ethan, Lysodren) is the only FDA (U.S. Food and Drug Administration) and EMA (European Medical Association) approved drug for treatment of ACC as palliative therapy administered as monotherapy or in combination with a cytotoxic chemotherapy regimen. In addition, it is recommended for adjuvant treatment for patients with a high-risk profile for recurrence (ENSAT stage III, R1 resection or Ki67 > 10%) (Hahner and Fassnacht, 2005, Fassnacht et al., 2013, Ronchi et al., 2014a, Fassnacht et al., 2018).

Derived from pesticide DDT, mitotane has a selective cytotoxic effect on the adrenal cortex by inhibition of proliferation of steroidogenic cells and hormone secretion (Bergenstal et al., 1959, Baudin et al., 2011, Ghataore et al., 2012, Hescot et al., 2013). Though mitotane has been in use for many years, the precise mechanism of action is still not completely understood. At a microscopic level, mitotane leads to destruction of both the zona fasciculata and reticularis while saving the zona glomerulosa, resulting in a

selective loss of ACTH controlled steroid secretion (Hart et al., 1973). On a cellular level, mitotane and its metabolites seems to mostly affect mitochondria (Cai et al., 1997, Hescot et al., 2013, Poli et al., 2013) and the endoplasmic reticulum (Sbiera et al., 2015) hindering the turnover of cholesterol into steroid hormones in ACC cells and leading to cytotoxicity through accumulation of toxic lipids. Pharmacokinetically, mitotane was found to act as CYP3A4 (cytochrome P450 3A4) inductor lowering the blood levels of several drugs as steroids, statins, anti-hypertensives, antibiotics, among others (Kroiss et al., 2011). It still remains unclear which enzymes are involved in mitotane metabolism, though a potential first-pass metabolism by CYP2B6 (cytochrome P450 2B6) was reported (D'Avolio et al., 2013).

About 40% of mitotane is absorbed from the gastrointestinal tract and a significant amount is distributed to fatty tissue, brain and liver and high dosage of mitotane is needed to achieve desired plasma levels. As a correlation between mitotane plasma levels and treatment efficacy was shown in patients with advanced ACC, mitotane concentrations between 14 and 20 mg/l are aimed at to achieve therapeutic response whilst minimizing toxic effects (Baudin et al., 2001, Hermsen et al., 2011). Adverse effects are common and mainly affect the gastrointestinal tract and the central nervous system (Hahner and Fassnacht, 2005, Daffara et al., 2008). Due to the adrenolytic effect of mitotane, consequent adrenal insufficiency has to be treated with a high dosage of hydrocortisone in all patients.

Several studies investigated the efficacy of mitotane in the clinical setting, however, due to the retrospective nature and small size of the cohorts, highly variable results were reported.

Overall, while mitotane treatment was associated with response rates of about 25% in patients with advanced ACC (Hahner and Fassnacht, 2005, Hermsen et al., 2011, Baudin et al., 2001, Gonzalez et al., 2007, Megerle et al., 2018), the benefit of adjuvant mitotane therapy is not definitely established yet as conflicting results were observed on this topic (Pommier and Brennan, 1992, Haak et al., 1994, Barzon et al., 1997, Grubbs et al., 2010), A large case-control study, however, could show significantly prolonged recurrence-free survival and OS (overall survival) in patients with completely resected ACC treated with adjuvant mitotane therapy in respect to an independent control group (Terzolo et al., 2007). In another study, it was proposed that only a subgroup of patients, e.g. with

cortisol-producing tumors, might benefit from mitotane treatment (Bertherat et al., 2007). Currently, a prospective multicentric international study has been performed to confirm these results (ADIUVO, NCT00777244). The study is now closed and data analyses are ongoing with the first results being awaited in 2020.

1.2.6.3. Chemotherapy

Cytotoxic chemotherapy is commonly administered to patients with aggressive recurrent and advanced ACC (Fassnacht et al., 2018). Combination with mitotane can increase the effectivity of certain chemotherapeutics, possibly due to reversing multidrug resistance by p-glycoprotein inhibition (Bates et al., 1991, Berruti et al., 2005). The effect of different chemotherapeutic regimens was investigated in several studies, the first prospective randomized trial being the FIRM-ACT (First International Randomized trial in locally advanced and Metastatic Adrenocortical Carcinoma Treatment) trial comparing two of the most promising regimens, EDP-M (etoposide, doxorubicin, cisplatin and mitotane) vs streptozotocin and mitotane. Observing a clear superiority of the EDP-M regimen regarding response and PFS (progression-free survival) as both first line and second line treatment, it was established as standard cytotoxic therapy for advanced ACC (Fassnacht et al., 2012).

However, with median PFS and OS even under EDP-M therapy being five and 15 months, respectively, and objective response rates of EDP-M being at about 23% (Fassnacht et al., 2012), there is an urgent need for new therapeutic options.

For patients progressing under EDP-M therapy a transition to second-line cytotoxic regimen as gemcitabine plus capecitabine with or without mitotane is suggested (Sperone et al., 2010, Henning et al., 2017). Another option is represented by streptozotocin plus mitotane (Khan et al., 2000, Fassnacht et al., 2012). Response rates are < 10% and PFS is < 4 months, however, and few patients did report significant benefit from treatment. Different approaches as trofosfamid (Kroiss et al., 2016, Berruti et al., 2012), paclitaxel (Berruti et al., 2012), docetaxel with cisplatin (Urup et al., 2013) and irinotecan (Baudin et al., 2002) showed disappointing results. Adjuvant chemotherapy is rarely administered with sparse data being available though it can be considered in patients with high risk for recurrence (Fassnacht et al., 2018).

1.2.6.4. Radiotherapy and other local therapeutic measures

Radiotherapy can be considered in an adjuvant setting in combination to mitotane (Cerquetti et al., 2008, Cerquetti et al., 2010) for patients with R1 (microscopic incomplete resection) and RX (uncertain resection) resection status or stage III tumors on an individualized basis, although the benefit is still controversial and only few studies with small cohort numbers are available (Fassnacht et al., 2018). While radiation showed capability of preventing local recurrence in most studies, efficacy on OS was generally poor (Fassnacht et al., 2006, Habra et al., 2013, Else et al., 2014b, Sabolch et al., 2015, Nelson et al., 2018). In a palliative setting, however, radiation is commonly used and has shown great benefit by reducing tumor mass, impeding hormone excess and pain relief, especially regarding bone metastases (Polat et al., 2009, Chow et al., 2012, Pin et al., 2018).

Other local therapeutic measures as RFA (radiofrequency ablation) (Ripley et al., 2011, Wood et al., 2003, Veltri et al., 2020) and TACE (transarterial chemoembolization) (Cazejust et al., 2010) can be used as individual palliative treatment strategy as an alternative to surgery in cases of rejection or contraindication of surgery. However, the efficacy of these methods has not been sufficiently evaluated yet in ACC patients.

1.2.7. Follow-up

The follow-up of ACC patients should include a regular physical examination, hormonal check-up and cross-sectional radiological imaging of abdomen, pelvis and chest (mostly CT scan). Patients with completely resected tumors are followed-up in three-month intervals for 2 years, and if patients are still recurrence-free intervals can be increased to six months. Surveillance intervals after 5 years of recurrence-free time can be further elongated. In patients with advanced ACC, follow-up intervals should be determined individually based on the current treatment and the prognosis and usually is around 2-3 months (Fassnacht et al., 2018, Else et al., 2014a).

1.2.8. Prognosis and prognostic markers

1.2.8.1. Clinical and histopathological markers

Prognosis in patients with ACC is generally poor with a median OS of about 3-4 years, but also highly variable dependent on clinical and histopathological features. A major

prognostic factor is the initial ENSAT tumor stage with a 5-year survival ranging from 82% for stage I to 18% for stage IV tumors (Fassnacht et al., 2009). Moreover, tumor resection status has a great impact on clinical outcome with R0 (complete resection) being associated with better survival (Bilimoria et al., 2008). A study focusing on ACC relapses found time to first recurrence in addition to resection status to be a good predictor of survival for recurrent tumors (Erdogan et al., 2013). Another important prognostic factor is the Ki67 proliferation index, as high Ki67 correlates with both worse recurrence-free survival after R0 resection and worse OS (Beuschlein et al., 2015). Similarly, grading tumors by mitotic count is considered to help define prognosis (Miller et al., 2010).

Additionally, age and hypercortisolism have been reported to influence survival in patients with complete resection (Abiven et al., 2006, Berruti et al., 2014, Vanbrabant et al., 2018, Libe et al., 2015). A GRAS score was proposed consisting of pathological grading (Weiss score, Ki67 percentage), resection status, age and symptoms due to autonomous steroid secretion or tumor mass (Libe et al., 2015) to predict prognosis in patients with advanced ACC.

Despite the high significance of these prognostic factors, prediction is still not perfectly possible for a small proportion of patients as, for example, about 20% of patients die within the first two years after complete resection and about 10% of patients survive more than 5 years even with metastasized ACC. Thus, identifying prognostic markers using molecular analysis might help to improve prognostic accuracy for these patients. A modified GRAS score (mGRAS score) was proposed by Lippert *et al*. (Lippert et al., 2018) including tumor stage, grading (Ki67 proliferation index), resection status, age and clinical symptoms.

1.2.8.2. Prognostic biomarkers

In addition to clinical and histopathological prognostic factors mentioned before, genomic analyses have stated that several molecular criteria can help to more accurately predict prognosis in ACC patients. For instance, gene expression analysis identified a subtraction of the RNA expression levels of *BUB1B* (BUB1 mitotic checkpoint serine/ threonine kinase B) and *PINK1* (phosphate and tensin homolog induced kinase 1) as prognostically significant (de Reynies et al., 2009).

Moreover, two large studies conducting genomic characterization of ACC described a stratification of ACC patients in molecular subgroups with different clinical outcome. Subgroups were identified by e.g. investigation of methylation status, CN (copy number) variations, miRNA profile and oncogenic alterations (Assie et al., 2014, Zheng et al., 2016). In consideration of clinical applicability, Lippert *et al.* (Lippert et al., 2018) developed a prognostic score combining the mGRAS score with a simplified molecular score including the number of somatic mutations, alterations in the Wnt/β-catenin pathway and promotor methylation status (COMBI score). Prognostic discrimination by the resulting COMBI score was found superior to each score itself. Moreover, targeted molecular classification was described as an independent prognostic marker of recurrence in localized ACC and a combination of tumor stage, tumor proliferation index and molecular classification was suggested to improve prognosis in ACC patients (Assie et al., 2019).

1.2.9. Precision medicine

Precision medicine is an algorithm-based targeted approach to management of a disease based on molecular diagnostic by genetic analysis of patients aiming to identify potentially targetable alterations, pre-select optimal treatment options and determine prognosis. This concept is particularly significant for cancer patients as tumors have variable underlying genetic causes while standard treatment take effect only in a subset of patients (Krzyszczyk et al., 2018).

1.2.9.1. Targeted therapy and ACC

Targeted therapy refers to a more specific way of identifying and attacking cancer cells by blocking the action of specific oncogenic genes or proteins or by modifying immune response. Due to its specificity, targeted therapy is presumably less harmful to normal cells and causes fewer adverse effects than conventional cancer treatment modalities.

1.2.9.1.1. Molecular drug targets

Three previous studies focused on identifying potential targetable molecular events in ACC tumors. De Martino *et al*. (De Martino et al., 2013) used hot spot gene sequencing and comparative genomic hybridization including 46 and 130 genes, respectively, while

Ross *et al*. (Ross et al., 2014) performed next generation sequencing in 29 ACC samples including 237 cancer-related genes and 47 introns. Both screening studies were performed with fresh-frozen tumor samples of up to 40 patients, whilst a recent study investigated 107 patients by targeted next-generation sequencing on paraffin-embedded ACC specimen (Lippert et al., 2018) aiming at an easily applicable method to be translated to clinical routine in the near future. Overall, at least one targetable molecular event was described in about 50-60%, mainly comprising members of cell cycle (e.g. *CDK* (cyclin-dependent kinases) *4* and *6* and *CDKN2A* (cyclin-dependent kinase inhibitor 2A), tyrosine kinase receptors (e.g. *EGFR* (epidermal growth factor receptor)*, PDGFRB* (platelet derived growth factor receptor beta)*, KIT* (tyrosine-protein kinase kit)) and the DNA repair system (e.g. *TP53, ATM* (ataxia telangiectasia mutated), *BRCA* (breast cancer type) *1* and *2*). While drugs targeting the p53 and the Wnt/β-catenin pathway are still in early development (Doghman et al., 2008, Demeure et al., 2011, Gaujoux et al., 2013), several targeted therapies for ACC patients have been evaluated in the last years. However, results have been mostly disappointing so far. Among the investigated targets were PDGFR and KIT, EGFR, VEGF and its pathway, mTOR (mechanistic target of rapamycin) and multi-tyrosine kinases (table 1.2). *IGF2* is well-known to be overexpressed in ACC tumors and *in vitro* studies could confirm a growth-promoting role of IGF2 and show good effects on cell viability by IGF2 inhibition (Guillaud-Bataille et al., 2014, Barlaskar et al., 2009, Peixoto Lira et al., 2016). Linsitinib (OSI-906), a dual inhibitor of IGF1R (insulin-like growth factor 1 receptor) and IR (insulin receptor), has been the only drug to enter phase III clinical trial for ACC that included 90 patients. While a partial response was observed in three patients, no increase in PFS or OS was documented (Fassnacht et al., 2015). It is of note, however, that the molecular background of included patients in this trial was largely unknown. More recent trials include the use of PLK1 (polo-like kinase 1) inhibitors (Demeure et al., 2016) as well as immunotherapy (Le Tourneau et al., 2018, Raj et al., 2019).

Failure of targeted therapy in ACC is an intensely discussed topic in literature (Drelon et al., 2013, Ronchi et al., 2014a, Kerkhofs et al., 2015). Firstly, it has to be considered that most patients included into these studies were heavily pre-treated with mitotane and chemotherapy. This might have led to treatment-resistance of the tumor. Drug interaction might also play an important role as the exact mechanism of action of mitotane remains

unclear although it was detected to acts as an CYP3A4 inductor (Kroiss et al., 2011). Even after finishing of mitotane treatment, plasma levels can remain high for a long time period. Secondly, targeting single pathways might cause compensatory hyper-activation of other signaling pathways as was observed in monotherapies with tyrosine kinase inhibitors. Thirdly, aside from linsitinib, studies were performed in small series of patients and therefore may lack significance. In addition, no information about the genetics of the specific ACC tumors was present. As targeted therapy using antibodies is most effective in tumors with a correspondent mutation, a genetic analysis of the treated patient may help to clarify.

Table 1.2: Targeted therapy approaches in ACC patients.

target	**drug**	**n**	**study phase**	**response (RECIST)**	**author**
tyrosine kinase					
PDGFR + KIT	imatinib	4	II	none	Gross et al., 2006
EGFR	gefinitib	19	II	none	Samnotra et al., 2007
EGFR + cytotoxic chemotherapy	erlotinib gemcitabine	10	II	10% SD	Quinkler et al., 2008
VEGF + cytotoxic chemotherapy	bevacizumab capecitabine	10	II (case series)	none	Wortmann et al., 2010
tyrosine kinases (VEGF pathway) + cytotoxic chemotherapy	sorafenib paclitaxel	9	II	none	Berruti et al., 2012
tyrosine kinases	sunitinib	35	II	14% SD	Kroiss et al., 2012
FGFR	dovitinib	17	II	6% PR 23% SD	García-Donas et al., 2014
VEGFR tyrosine kinases	axitinib	13	II	31% SD	O'Sullivan et al., 2014

Multi tyrosine kinases	cabozatinib	16	II	19% PR 31% SD	Kroiss et al., 2020
IGF1R/mTOR					
IGF1R	figitimumab	14	I	57% SD	Haluska et al., 2010
mTOR	everolismus	4	II	none	Fraenkel et al., 2013
mTOR + immunomodulation	temsirolismus lenalidomide	3	I	33% SD	Ganesan et al., 2013
IGF1R mTOR	cixutumumab temsirolismus	26	I	42% SD	Naing et al., 2013
IGF1R + adrenolytic drug	cixutumumab mitotane	20	II	5% PR 35% SD	Lerario et al., 2014
IGF1R + IR	linsitinib	90	III	6.7% SD 3.3% PR	Fassnacht et al., 2015
cell cycle					
PLK1	TKM-080301	8	I/II	13% PR 50% SD	Demeure et al., 2016
immunomodulation					
PD-L1	avelumab	50	I	6% PR 42% SD	Le Tourneau et al., 2018
PD-1 receptor	nivolumab	10	II	29% SD	Carneiro et al., 2019
PD-1 receptor	pembrolizumab	14	II	14% PR 50% SD	Habra et al., 2019
PD-1 receptor	pembrolizumab	39	II	23% PR 18% SD	Raj et al., 2019

Abbreviations: PDGFR = platelet-derived growth factor receptor; KIT = tyrosine-protein kinase kit; EGFR = epidermal growth factor receptor; VEGF = vascular endothelial growth factor; FGFR = fibroblast growth factor receptor; VEGFR = vascular endothelial growth factor receptor; IGF1R = insulin-like growth factor 1 receptor; IR = insulin receptor; mTOR = mechanistic target of rapamycin; PLK1 = polo-like kinase 1; PD-L1 = programmed death ligand 1; PD-1 = programmed cell death protein 1; RECIST = response evaluation criteria in solid tumors; SD = stable disease; PR = partial response.

1.2.9.1.2. [^{131}]iodometomidate radionuclide therapy

Another approach to target adrenal cancer is a radionuclide therapy based on [^{131}]iodometomidate, radiolabeled metomidate, which has shown high specific tracer uptake in ACC originated tissue by binding to the 11-beta hydroxylase and aldosterone synthase providing a possibility of targeted radionuclide therapy (Hahner et al., 2012). Five of eleven examined patients with advanced ACC experienced stable disease and one partial response.

1.2.9.2. Predictive biomarkers

Predictive markers could help to improve determination of appropriate therapeutic options for ACC patients, as both mitotane and the EDP regimen are associated with low response rates and severe adverse effects. Despite this, only few reliable molecular markers predictive for response to treatment have been proposed yet for ACC. For mitotane treatment, high CYP2W1 (cytochrome P450 2W1) protein expression was found to correlate with longer TTP (time to progression) and OS as well as better response to mitotane therapy (Ronchi et al., 2014b). Similarly, a high SOAT1 (sterol O-acyltransferase 1) expression was associated with longer TTP as well as better response rates (Sbiera et al., 2015). However, this could not be confirmed in a larger study (Weigand et al., 2020). High RRM1 (ribonucleotide reductase catalytic subunit M1) gene expression, in contrast, was observed in relationship with impaired disease-free survival and OS, possibly due to its interference with mitotane metabolism as shown in adrenocortical cancer cells (Volante et al., 2012, Germano et al., 2015). TOP2A (topoisomerase 2-alpha) and TS (thymidylate synthase) have been demonstrated to be predictive markers for anthracyclines, topoisomerase inhibitors and fluoropyrimidines in different tumor entities. Therefore, their predictive potential was investigated for the cytotoxic chemotherapy regimen EDP in ACC patients, and high TOP2A expression was detected to favorably impact EDP-M response and on TTP (Roca et al., 2017). Furthermore, high ERCC1 (excision repair cross-complementation group 1) protein expression known to predict resistance to platinum compounds in several tumors was found to be associated with worse OS after platinum treatment (Ronchi et al., 2009), however, this effect did not sustain when investigated in a larger cohort (Laufs et al., 2018). Resistance to gemcitabine was described in tumor patients with hENT1 (human

equilibrative nucleoside transporter type 1) and/or RRM1 expression, however, no similar relationship was reported in gemcitabine-treated ACC patients (Henning et al., 2017). Overall, despite some initial and potentially promising findings on predictive markers, none was validated yet.

1.3. Objectives of this thesis

ACC are rare and aggressive tumors with limited therapeutic options for advanced stages. In the past years, genomic characterization has led to a better understanding of adrenal tumors pathogenesis, however, no effective targeted therapies have emerged. Moreover, methods utilized for past pan-genomic studies on ACC are still hardly transferable to routine application.

While whole transcriptome sequencing offers a broad range of opportunities and insights, targeted RNA profiling is a more cost- and time-effective approach to focus on genes of interest representing an easier method to be integrated to clinical routine. Similarly, the use of routinely available FFPE (formalin-fixed paraffin embedded) samples can improve the application to the clinical setting despite a lower yield of good quality RNA in comparison to snap-frozen tissue as was used in past studies.

Thus, the main objective of this thesis was to identify potential new drug targetable events and targeted treatment strategies with methods easily applicable to the clinical setting. In particular, this thesis addresses following questions:

1. Is targeted RNA sequencing feasible from archived FFPE samples of ACC? Can this method identify possible drug targets?
2. Does RNA expression of selected drug target candidates through targeted RNA sequencing correlate with protein expression?
3. Do selected drug targets through targeted RNA sequencing affect cell viability and can they be sufficiently targeted by corresponding available inhibitors? Is a combination of different available inhibitors possible?

Therefore, targeted RNA profiling was carried out in a large series of ACC using routinely available FFPE specimen. The protein expression of best candidate drug targets was then investigated by IHC (immunohistochemistry) and the efficacy of corresponding available inhibitors was tested by functional *in vitro* experiments in ACC cell lines.

This thesis was embedded in a research project aiming to define molecular events characteristic to ACC with impact on prognosis or response to therapy as well as to identify new drug targets. The expected results were intended to be integrated into an ACC-specific panel for risk stratification and personalized management (i.e. personalized therapeutic approach).

An outline of the design of this research project is shown in figure 1.

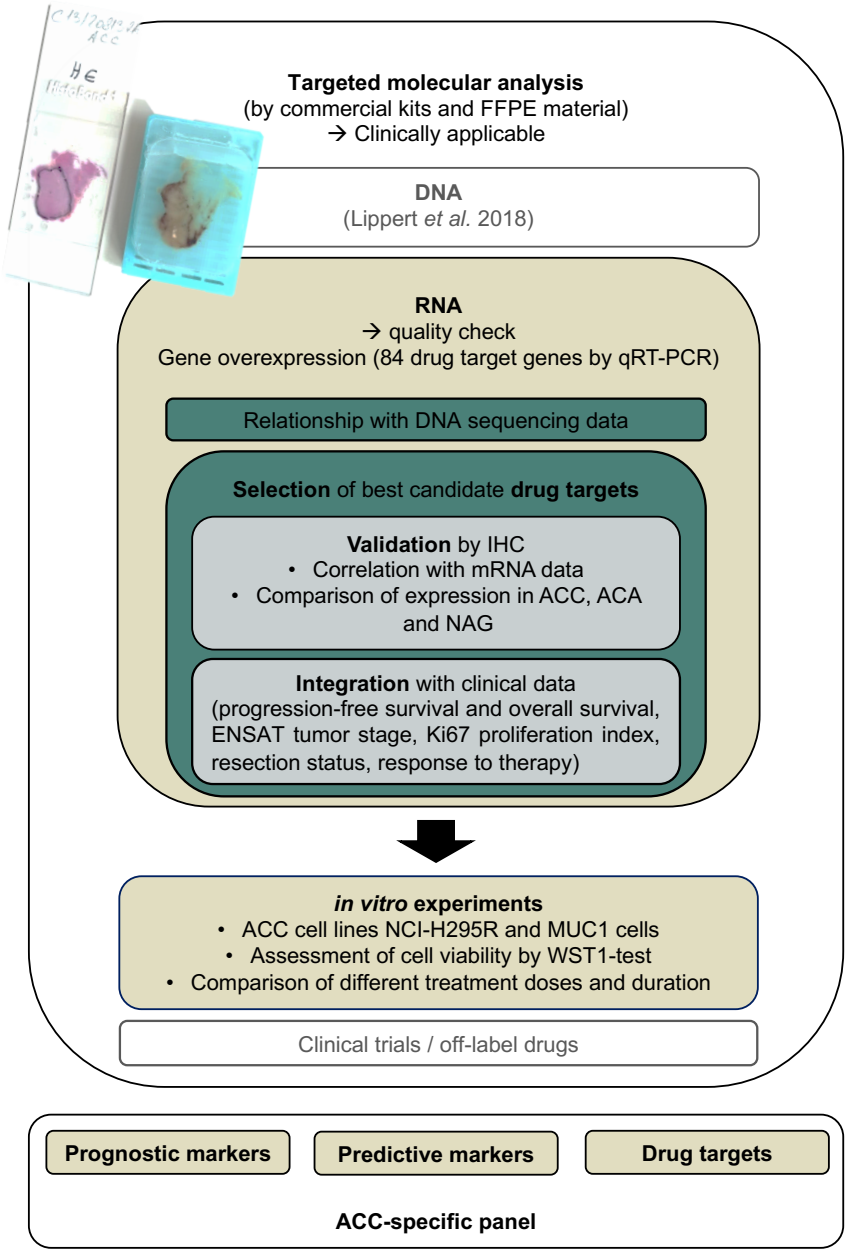


Figure 1: Outline of the research project conducted for this thesis. Overall, an ACC specific panel was obtained through targeted molecular analyses. Selected drug target candidates were validated through IHC and investigated in ACC cell lines. Main elements of this thesis are presented in black font, other elements of this project not conducted in this thesis are presented in grey font.

2. Material and methods

2.1. Patient cohort and tumor material

For the present study, a total of 107 patients with histologically confirmed diagnosis of ACC examined in a previous project on targeted genetic analysis and availability of DNA sequencing data (Lippert et al., 2018) were taken into consideration. Additional FFPE tumor material collected between 2002 and 2016 was available for 104/107 patients (87 primary tumors, 9 local recurrences and 8 distant metastases) forming the final cohort for this study. Amongst them, the female to male ratio was 1.3:1 with the median age being 49 years. About 20% of tumors showed secretion of either cortisol, mixed steroids or no steroid secretion with only a minority of tumors being associated with steroid secretion other than cortisol. About half of the patients were diagnosed in an early tumor stage (ENSAT tumor stage I and II) and in the majority of patients (69%) R0 resection was possible. The Ki67% index showed a median value of 15 with a range between 2 and 90. In terms of pharmacological therapy, over a third of patients received mitotane in an adjuvant setting and a similar number in a palliative setting. About half of the patients were treated with a platinum-based chemotherapy regimen, while 41% of them received streptozotocin and 37% gemcitabine plus capecitabin.

From the total cohort a subgroup suitable for mRNA analyses according to the quality of extracted RNA (mRNA cohort) consisting of 40 patients (33 primary tumors, 5 local recurrences and 2 distant metastases) was established with similar clinical and histopathological characteristics as the total cohort. Relevant clinical and histopathological characteristics for the entire and mRNA cohort are listed in table 2.1.

All FFPE specimens were appropriately stored at RT (room temperature) at the Division of Endocrinology and Diabetology protected from light.

Baseline clinical and histopathological characteristics and follow up information were available for all patients and collected through the ENSAT registry (https://registry.ensat.org//) as well as through medical reports, tumor board protocols as well as pathology and radiology reports stored in the hospital information system of the university hospital of Wuerzburg.

The study protocol was approved by local ethics committee (#88/11) and written informed consent was obtained from all subjects prior to study enrollment.

Table 2.1: Clinical and histopathological characteristics of patients with ACC in the entire cohort and in the subgroup used for mRNA expression analysis (mRNA cohort) (Liang et al., 2020)**.**

	Total cohort	mRNA cohort
n	**104**	**40**
Sex (F/M)	59/45	25/15
Baseline		
Age – yrs (median, range)	49 (18-87)	46 (18-81)
< 50 years – *n (%)*	54 (51.9)	24 (60.0)
≥ 50 years – *n (%)*	50 (48.1)	16 (40.0)
Steroid secretion – *n* available	78	33
Cortisol – *n (%)*	23 (22.1)	9 (22.5)
Other single steroids (androgens, mineralocorticoids, or estrogens) – *n (%)*	9 (8.7)	4 (10.0)
Mixed steroids – *n (%)*	21 (20.2)	9 (22.5)
Inactive – *n (%)*	25 (24.0)	11 (27.5)
Initial ENSAT tumor stage		
I-II – *n (%)*	55 (52.9)	21 (52.5)
III – *n (%)*	27 (26.0)	14 (35.0)
IV – *n (%)*	22 (21.1)	5 (12.5)
Resection status of first surgery – *n* available	101	40
R0 – *n (%)*	72 (69.2)	28 (70.0)
RX – *n (%)*	16 (15.4)	7 (17.5)
R1 – *n (%)*	5 (4.8)	3 (7.5)
R2 – *n (%)*	8 (7.7)	2 (5.0)
Ki67% index – median (range)	15 (2-90)	17.5 (3-90)
Tumor localization		
Primary tumor – *n (%)*	87 (83.7)	33 (82.5)
Local recurrences – *n (%)*	8 (7.7)	5 (12.5)
Metastases – *n (%)*	9 (8.6)	2 (5.0)
Follow-up		
Duration of follow up – months (median, range)	36 (1-280)	31 (4-280)
Deaths	53 (51.0)	18 (45.0)
Therapeutic approaches		
Additional surgeries – *n (%)*	38 (36.5)	18 (45.0)
Radiotherapy (tumor bed or metastases) – *n (%)*	32 (30.8)	15 (37.5)
Mitotane		
Adjuvant setting – *n (%)*	38 (36.5)	16 (40.0)
Palliative setting – *n (%)*	38 (36.5)	14 (35.0)
Cytotoxic chemotherapies		
None – *n (%)*	41 (39.4)	17 (42.5)
Platinum-based regimen – *n (%)*	53 (51.0)	18 (45.0)
Streptozotocin – *n (%)*	43 (41.3)	19 (47.5)
Gemcitabin plus capecitabin – *n (%)*	37 (35.6)	14 (35.0)
Iodometomidate – *n (%)*	4 (3.8)	4 (10.0)

Abbreviations: F = female; M = male; n = number of patients; R0 = complete resection; R1 = microscopic incomplete resection; R2 = macroscopic incomplete resection; RX = uncertain resection; yrs = years.

2.2. Material

2.2.1. Kits, reagents, chemicals and consumables

Specific kits, reagents, chemicals and consumables used during this thesis are listed in table 2.2. Standard chemicals not further specified were obtained from Applichem, Bio-Rad, Merck, Roth and Sigma-Aldrich. All laboratory disposables were obtained from A. Hartenstein, Corning, Eppendorf, Greiner, Bio-One and Sarsted.

Table 2.2: Kits, reagents and chemicals used during this thesis.

Name	Application	Manufacturer
AB serum	IHC	Sigma-Aldrich
Advance HRP Link & Enzyme	IHC	Dako
BCA Kit	protein quantification	Sigma-Aldrich
Cell Proliferation Reagent WST1	cell proliferation quantification	Sigma-Aldrich
ECL Prime Western Blotting Detection Reagents	WB	GE Healthcare
Liquid DAB+ Substrate Chromogen System	IHC	Dako
Maxwell RSC simplyRNA Tissue Kit	RNA isolation	Promega
Mini-PROTEAN TGX Precast Gels	SDS-PAGE	Bio-Rad
miRNeasy FFPE Kit	RNA isolation	QIAGEN
Phosphate Inhibitor Cocktail B	cell lysis	Santa Cruz
Phosphate Inhibitor Cocktail C	cell lysis	Santa Cruz
Protease Inhibitor Cocktail	cell lysis	Sigma-Aldrich
PageRuler Plus Prestained Protein Ladder	SDS-PAGE	Thermo Fisher
Proteinase K	cell lysis	Promega
RIPA buffer	cell lysis	Sigma-Aldrich
RT² First Strand Kit	RT-qPCR	QIAGEN

RT² Profiler PCR Array Human Cancer Drug Targets	RT-qPCR	QIAGEN
RT² SYBR Green qPCR Mastermix	RT-qPCR	QIAGEN
TaqMan Gene Expression Master Mix	RT-qPCR	Thermo Fisher
QuantiTect Reverse Transcription Kit	RT-qPCR	QIAGEN

Abbreviations: HRP = horseradish peroxidase; BCA = bicinchoninic acid kit; WST1 = water soluble tetrazolium 1; ECL = enhanced chemiluminescence; DAB = 3,3'-diaminobenzidine; RNA = ribonucleic acid; FFPE = formalin-fixed paraffin embedded; RIPA = radioimmunoprecipitation assay buffer; IHC = immunohistochemistry; WB = western blot; RT-qPCR = quantitative reverse transcription polymerase chain reaction; SDS-PAGE = sodium dodecyl sulfate polyacrylamide gel electrophoresis.

2.2.2. Equipment

The equipment used during this thesis is listed in table 2.3.

Table 2.3: Equipment used during this thesis.

device	**Type and manufacturer**
blotting chamber	Mini PROTEAN II Bio-Rad Laboratories GmbH, Munich, Germany
camera	AxioCam MRm, Carl Zeiss AG, Jena, Germany
cell counter	Countess II FL Life Technologies, Waltham, MA, USA
centrifuges	Mikro 200R, Andreas Hettich GmbH & Co.KG, Tuttlingen, Germany Megafuge 1.0R, Heraeus Holding GmbH, Hanau, Germany
CO_2 incubator	C150 Binder GmbH, Tuttlingen, Germany
cycler	Mastercycler personal, Eppendorf AG, Hamburg, Germany
extraction robot	Maxwell RSC Promega GmbH, Mannheim, Germany
microscopes	Axiovert 135 Carl Zeiss AG, Jena, Germany Primo Vert Carl Zeiss AG, Jena, Germany
microtome	Slee Cut 5062, Mainz, Germany
mini rotator	A. Hartenstein, Wuerzburg, Germany
microplate reader	1420 Victor3 PerkinElmer Inc., Waltham, MA, USA

PAGE apparatus	Mini PROTEAN Tetra Cell, Bio-Rad Laboratories GmbH, Munich, Germany
pH meter	inoLab, A. Hartenstein, Wuerzburg, Germany
photometer	BioPhotometer plus, Eppendorf AG, Hamburg, Germany
power supply	PowerPac Basic Bio-Rad Laboratories GmbH, Munich, Germany
pipet gun accu-jet pro	Brand GmbH & Co. KG, Wertheim, Germany
platform shaker	L-40, A. Hartenstein, Wuerzburg, Germany
qPCR cycler	CFX96 BioRad Laboratories GmbH, Munich, Germany
sonifier	Sonopuls GM70, Bandelin electronic GmbH & Co. KG, Berlin, Germany
thermo block	DB.2D Dri-Block, Techne, Staffordshire, UK
thermo shaker	TS-100 A. Hartenstein, Wuerzburg, Germany
UV spectrophometer	Peqlab Nanodrop 2000c, Thermo Fisher Scientific, Waltham, MA, USA
vortex mixer	Uzusio VTX-3000L, LMS, Tokyo, Japan

Abbreviations: PAGE = polyacrylamide gel electrophoresis; qPCR = quantitative real-time polymerase chain reaction.

2.3. Methods

2.3.1. Molecular biological methods

2.3.1.1. RNA isolation

2.3.1.1.1. RNA isolation from FFPE tissue

RNA was isolated from 104 ACC and 5 NAG samples by the RNeasy FFPE Kit (Qiagen) for quantification of mRNA expression. HE-staining was performed beforehand to assess the tumor-cell content of each FFPE sample (median 90%, range 60-95%). Depending on the size of the tumor, five to seven slides of 2 µm thick FFPE tissue were used per sample. All further steps were carried out according to manufacturer's instruction.

2.3.1.1.2. RNA isolation from snap-frozen tissue

To validate results of RNA isolation from FFPE, RNA was isolated from five snap-frozen tissue (matched to FFPE samples) by Maxwell RSC simplyRNA Tissue Kit (Promega). About 30 mg tissue was mixed with a previously prepared 400 µl

thioglycerol/homogenization buffer to achieve homogenization by ball mill and ultrasonic homogenizer. Further processing occurred according to manufacturer's instructions.

2.3.1.1.3. RNA isolation from cells

RNA extraction from cells was performed using the Maxwell RSC simplyRNA Tissue Kit (Promega). Cells of a 6-well plate were detached with 200 µl of previously prepared thioglycerol/homogenization buffer and transferred to a 1.5 ml reaction tube. Further processing occurred according to manufacturer's instructions.

2.3.1.1.4. Concentration measurement and storage of obtained RNA

Extracted RNA concentration was measured with the Nanodrop spectrometer in duplicates and all RNA samples were appropriately stored at -80 °C.

2.3.1.2. cDNA transcription

2.3.1.2.1. cDNA transcription for RT-qPCR (real-time quantitative RT-PCR) with Taqman probes

For cDNA transcription, the QuantiTect Reverse Transcription Kit (Qiagen) was used according to manufacturer's instructions. Nuclease-free water was added to 1 µg RNA achieving a total volume of 24 µl and then mixed with 4 µl wipeout buffer. After incubation at 42 °C for 5 min, the reverse transcription mix was added. Cycler conditions were as listed below to allow cDNA synthesis.

cDNA reverse transcription mix

2 µl RT Primer-Mix
8 µl 5x Quantiscript RT-Buffer
2 µl Quantiscript Reverse Transcriptase

cDNA cycling conditions

42 °C for 15 min
95 °C for 3 min
4 °C ∞

2.3.1.2.2. First strand transcription for targeted mRNA profiling

1 µg RNA was transcribed with the RT^2 First Strand Kit (Qiagen) adding nuclease-free water to obtain a total volume of 8 µl. This mixture was combined with 2 µl of genomic DNA elimination mix. After incubation at 42 °C for 5 min, the reverse transcription mix was added and then incubated in the cycler according to manufacturer's instructions. Cycler conditions were as listed below. Shortly before use for targeted mRNA profiling, cDNA was diluted with 91 µl nuclease-free water.

First strand reverse transcription mix

4 µl 5x Buffer BC3
1 µl Control P2
2 µl RE3 Reverse Transcriptase Mix
3 µl nuclease-free water

First strand cycling conditions

42 °C for 15 min
95 °C for 5 min
4 °C ∞

2.3.1.2.3. Storage of obtained cDNA

All cDNA samples were appropriately stored at -20 °C.

2.3.1.3. Quantitative polymerase chain reaction

2.3.1.3.1. RT-qPCR

mRNA expression levels of the genes of interest in tumor tissue or cells were assessed by RT-qPCR using TaqMan probes. All TaqMan probes were purchased from Thermo Fisher Scientific. The corresponding probe numbers are as follows:

- *β-actin*: Hs99999903_m1
- *CCND1*: Hs00765553_m1
- *CDK1*: Hs00938777_m1
- *CDK4*: Hs00364847_m1
- *CDK6*: Hs01026371_m1

- *CDKN2A*: Hs00923894_m1
- *GAPDH*: Hs99999905_m1
- *RB1*: Hs01078066_m1

First of all, the quality of FFPE-derived RNA was determined by the two housekeeping genes *ACTB* and *GAPDH* excluding samples with a CT > 39 from further analysis. Consequently, 40 out of 104 specimens qualified for further analysis (mRNA cohort, see table 2.1) and were transcribed with the RT2 First Strand Kit for further investigation by targeted mRNA analysis. Moreover, RT-qPCR was used to analyze mRNA expression levels of genes related to the CDK4 pathway in ACC cell lines.

The RT-qPCR reaction mix (Thermo Fisher) was added to 2 µl cDNA for each sample in a 96-well PCR plate, each sample was analyzed in duplicates. RT-qPCR was performed on the CFX96 real-time thermocycler using the Bio-Rad CFX Manager 2.0 software (Bio-Rad) and cycling conditions as listed below. Gene expression levels were then calculated with the ΔCT method and normalized to β-actin.

RT-qPCR reaction mix

12.5 µl TaqMan Gene Expression Mastermix

1.25 µl pre-designed TaqMan probe

9.25 µl nuclease-free water

RT-qPCR cycling conditions

95 °C for 3 min

Followed by 49 cycles of:

95 °C for 30 sec

60 °C for 30 sec

72 °C for 30 sec

2.3.1.3.2. Targeted mRNA profiling

Out of 104 examined cases, a total of 40 ACC samples (mRNA cohort) presented a CT value of ≤ 39 when tested for mRNA expression levels of *ACTB* and *GAPDH* and therefore qualified for further investigation by targeted mRNA profiling. Using the

Human Cancer Drug Targets RT2 Profiler PCR Array (Qiagen) mRNA expression of 84 targetable genes suspected or known to be related to tumorigeneses, five housekeeping genes (*ACTB*, *B2M*, *GAPDH*, *HPRT1*, *RPLPO*) and seven positive controls were analyzed as seen for an exemplary sample in figure 2.1.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Unk 17214_12 ABCC1	Unk 17214_12 AKT1	Unk 17214_12 AKT2	Unk 17214_12 ATF2	Unk 17214_12 AURKA	Unk 17214_12 AURKB	Unk 17214_12 AURKC	Unk 17214_12 BCL2	Unk 17214_12 BIRC5	Unk 17214_12 CDC25A	Unk 17214_12 CDK1	Unk 17214_12 CDK2
B	Unk 17214_12 CDK4	Unk 17214_12 CDK5	Unk 17214_12 CDK7	Unk 17214_12 CDK8	Unk 17214_12 CDK9	Unk 17214_12 CTSB	Unk 17214_12 CTSD	Unk 17214_12 CTSL	Unk 17214_12 CTSS	Unk 17214_12 EGFR	Unk 17214_12 ERBB2	Unk 17214_12 ERBB3
C	Unk 17214_12 ERBB4	Unk 17214_12 ESR1	Unk 17214_12 ESR2	Unk 17214_12 FIGF	Unk 17214_12 FLT1	Unk 17214_12 FLT4	Unk 17214_12 GRB2	Unk 17214_12 GSTP1	Unk 17214_12 HDAC1	Unk 17214_12 HDAC11	Unk 17214_12 HDAC2	Unk 17214_12 HDAC3
D	Unk 17214_12 HDAC4	Unk 17214_12 HDAC6	Unk 17214_12 HDAC7	Unk 17214_12 HDAC8	Unk 17214_12 HIF1A	Unk 17214_12 HRAS	Unk 17214_12 HSP90AA1	Unk 17214_12 HSP90B1	Unk 17214_12 IGF1	Unk 17214_12 IGF1R	Unk 17214_12 IGF2	Unk 17214_12 IRF5
E	Unk 17214_12 KDR	Unk 17214_12 KIT	Unk 17214_12 KRAS	Unk 17214_12 MDM2	Unk 17214_12 MDM4	Unk 17214_12 MTOR	Unk 17214_12 NFKB1	Unk 17214_12 NRAS	Unk 17214_12 NTN3	Unk 17214_12 PARP1	Unk 17214_12 PARP2	Unk 17214_12 PARP4
F	Unk 17214_12 PDGFRA	Unk 17214_12 PDGFRB	Unk 17214_12 PGR	Unk 17214_12 PIK3C2A	Unk 17214_12 PIK3C3	Unk 17214_12 PIK3CA	Unk 17214_12 PLK1	Unk 17214_12 PLK2	Unk 17214_12 PLK3	Unk 17214_12 PLK4	Unk 17214_12 PRKCA	Unk 17214_12 PRKCB
G	Unk 17214_12 PRKCD	Unk 17214_12 PRKCE	Unk 17214_12 PTGS2	Unk 17214_12 RHOA	Unk 17214_12 RHOB	Unk 17214_12 TERT	Unk 17214_12 TNKS	Unk 17214_12 TOP2A	Unk 17214_12 TOP2B	Unk 17214_12 TB53	Unk 17214_12 TXN	Unk 17214_12 TXNRD1
H	Unk 17214_12 ACTB	Unk 17214_12 B2M	Unk 17214_12 GAPDH	Unk 17214_12 HPRT1	Unk 17214_12 RPLPO	Unk 17214_12 HGDC	Unk 17214_12 RTC	Unk 17214_12 RTC	Unk 17214_12 RTC	Unk 17214_12 PPC	Unk 17214_12 PPC	Unk 17214_12 PPC

Figure 2.1: Example of a RT-qPCR run for one specimen using the Human Cancer Drug Targets RT2 Profiler PCR Array (Qiagen). The 84 cancer drug target genes are displayed in the top seven rows (A-G) outlined in black, the bottom row (F) shows five housekeeping genes (1-5) and seven positive control genes (6-12).

For targeted mRNA profiling, 102 µl cDNA was combined with 1.350 ml RT2 SYBR Green qPCR Mastermix (Qiagen) and 1.248 ml nuclease-free water, the reaction ran on the CFX96 real-time thermocycler using the Bio-Rad CFX Manager 2.0 software and cycling conditions as listed below. FC (fold change) was calculated with the 2^(-ΔΔCT) method and normalized to five housekeeping genes as well as to a pool of five NAG on FFPE specimens as reference by the Qiagen GeneGlobe Data Analysis Center (https: // www.qiagen.com/de/shop/genes-and-pathways/data-analysis-center-overview - page).

Targeted RT-qPCR cycling conditions

95 °C for 10 min

Followed by 40 cycles of:

95 °C for 15 sec

60 °C for 60 sec

2.3.2. IHC

The most frequently overexpressed genes at targeted mRNA profiling were further assessed by IHC to validate the expression at protein level. IHC was performed on 104 FFPE tumor specimen of the investigated ACC cohort and an additional 11 ACA and 6 NAG. Other tissues such as normal tonsil and thyroid carcinoma served as external positive controls.

2.3.2.1. Microtome sectioning

FFPE tissue blocks were chilled at -20 °C 2 h prior to sectioning. Slices were cut at a thickness of 2 µm, floated on a water bath at 45 °C for flattening and then picked up by a specimen slide. The samples were dried overnight and stored at RT.

2.3.2.2. Immunohistochemical staining method

IHC was performed on 2 µm sections. After deparaffinization in Xylol two times for 12 min each and rehydration in 100% ethanol, 90% ethanol, 80% ethanol and 70% ethanol for 5 min each. Remaining alcohol was removed by 5 washing steps with distilled water. Antigen was retrieved by heating the slides for 13 min in the pressure cooker in a 10 mM citric acid monohydrate buffer (pH 6.5). The slides were allowed to cool down for 20 min and then rinsed with distilled water for 5 times. To inhibit the endogenous peroxidase the slides were covered in a 3% hydrogen peroxide-methanol-mix for 10 min and then washed for 5 times with distilled water. Binding sites were blocked with 20% AB-serum mixed with PBS (phosphate buffered saline) for 1 h minimizing unspecific antibody interaction. Slides were then incubated with a specific antibody diluted in PBS to the desired concentration for another hour while anti-mouse or anti-rabbit antibodies functioned as negative controls. The utilized antibodies and their individual dilutions as applied for this thesis are reported in table 2.4. Afterwards, 5 washing steps using PBS were performed. Antibody binding was detected by means of HRP (horseradish peroxidase) link and enzyme (Dako). HRP link was applied for 20 min and then washed 3 times for 5 min in PBS, these steps were repeated in the dark for HRP enzyme. After development with DAB (3,3'-diaminobenzidine) for 10 min, slides were washed three times with tab water and nuclei were counterstained with hematoxylin for 2 min and blued under running tab water for 5 min.

All steps were performed at RT and under humid conditions. Slides were then dried by dehydration with ethanol and incubation in a drying oven at 56 °C for 20 min. Mounting was achieved by entellan.
The antibodies and the composition of the buffers used for IHC can be found below.

Table 2.4: Antibodies utilized for IHC.

antigen	clone or number	host	Supplier	application and dilution
CDK1	ab18	mouse	Abcam	IHC (1:500)
CDK4	EPR4513-32-7	rabbit	Abcam	IHC (1:20)
PLK1	13E18	mouse	Thermo Fisher	IHC (1:50)
TOP2A	EP1102y	rabbit	Abcam	IHC (1:500)
universal negative control mouse	N1698	mouse	Dako	IHC (undiluted)
universal negative control rabbit	N1699	rabbit	Dako	IHC (undiluted)

Abbreviations: IHC = immunohistochemistry; CDK = cyclin-dependent kinase; PLK = polo-like kinase; TOP2A = topoisomerase 2 alpha.

Buffers utilized for IHC:

- **Citric acid monohydrate buffer**
  10 mM citric acid monohydrate buffer
  pH 6.5
- **PBS buffer**
  PBS tablet (Sigma-Aldrich)
  pH 7.6

2.3.2.3. Microscopy and analysis of FFPE tissue slides

All slides were evaluated by two independent operators blinded to the results and clinical information using the Scope A1 microscope (Carl Zeiss AG, Jena, Germany). Intensity of staining was rated negative (0), weakly positive (1), moderately positive (2) and strongly positive (3) and percentage of positive cells were determined by a proportion

score ranging from 0 if 0% were positive, 0.1 if 1-9% were positive, 0.5 if 10-49% were positive and 1 if ≥ 50% were positive. Multiplication of the staining intensity score and the proportion score resulted in the final H-score (Ronchi et al., 2009). In case of discrepancies, slides were jointly assessed by both investigators, forming a definite score by consensus. Inter-observer agreement was determined by Pearson correlation coefficients: 0.62 (95%CI (95% confidence intervals) = 0.49-0.72) for CDK1, 0.67 (95%CI = 0.55-0.76) for CDK4, 0.53 (95%CI = 0.39-0.65) for PLK1 and 0.79 (95%CI =0.71-0.85) for TOP2A. Photographs of slides were taken by the Axiocam 503 color (Carl Zeiss AG, Jena, Germany) and brightness and white balance were adjusted through Zen2 (Carl Zeiss AG, Jena, Germany).

2.3.3. Selection of drug target candidates

The most frequently overexpressed genes at targeted RT-qPCR arrays were further investigated to assess their potential as drug targetable events. Based on median FC values and frequency of overexpression defined as FC ≥ 2.0 in at least 50% of cases, six candidates were pre-selected. Considering the current status of research, two of these six candidates were excluded from further investigation on protein level. *IGF2* is well-known to be overexpressed in ACC and has already been extensively examined as a drug target both *in vitro* and in clinical trials while reports on *PLK4* are scarce and its functional roles in normal cell biology not yet clearly understood (Liu, 2015). Therefore, both genes were removed from consideration. The decisive criterion for selection of the suitable drug target candidate for drug testing in cell culture was the availability of specific inhibitors already approved by both FDA and EMA or at least in phase III clinical trials on solid tumors. The current stage of inhibitors targeting these gene candidates is shown in table 2.5. Accordingly, further investigation was focused on *CDK4* (cyclin-dependent kinase 4).

Table 2.5: Currently available inhibitors targeting the most frequently overexpressed genes reported in the present study.

Gene	Available inhibitors (examples)	Current stage
IGF2	IGF1R/IR inhibitor (e.g. linsitinib)	Phase III trial in ACC patients (OSI-906)[1]
TOP2A	TOP2A inhibitors (e.g. aclarubicin)	Preclinical studies in ACC cells[2]
CDK1	Pan-CDK inhibitors (e.g. flavopiridol)	Phase I/II trials ongoing in solid tumors Preclinical studies in ACC cells[3]
CDK4	CDK4/6 inhibitors (e.g. palbociclib)	FDA and EMA approved for HR-positive/ HER2 negative breast cancer Phase II trials in liposarcoma Preclinical studies in ACC cells[4]
PLK4	PLK4 inhibitor (fumarate)	Phase I trials ongoing in solid tumors
PLK1	PLK1 inhibitor (e.g. TKM-080301, volasertib, onvansertib, rigosertib)	Phase I/II trials ongoing in solid (including ACC[5]) and non-solid tumors

[1]Fassnacht et al., 2015; [2]Jain et al., 2013; [3]Nilubol et al., 2018; [4]Fiorentini et al., 2018, [5]Demeure et al., 2016.

IGF2 = insulin growth factor 2; TOP2A = topoisomerase 2 alpha; CDK = cyclin-dependent kinase; PLK = polo-like kinase; IGF1R = insulin growth factor 1 receptor; IR = insulin receptor; ACC = adrenocortical carcinoma; FDA = Food and Drug Association; EMA = European Medical Association; HR = hormone receptor; HER2 = human epidermal growth factor receptor 2.

Sources: www.clinicaltrials.com, www.ema.europa.eu/en/medicines, www.fda.gov/drugs/informationondrugs/approveddrugs

2.3.4. CDK4 as drug target

CDK4 encoding for the cyclin-dependent kinase 4, along with *CDK6*, is activated by binding to D-type cyclins and is responsible for the G1/S transition in the cell cycle by phosphorylation and inhibition of the RB protein together with the related proteins p107/RBL1 and p130/RBL2 (Sherr et al., 2016). Regulation occurs through its inhibitor *CDKN2A* respectively $p16^{INK4A}$. A schematic outline of the CDK4 pathway with available inhibitors is shown in figure 2.2.

Recently, several CDK4/6 inhibitors, such as palbociclib or abemaciclib, were approved for HR (hormone receptor)-positive/HER2 (human epidermal growth factor receptor 2)-negative breast cancer and are currently under investigation in clinical trials on solid tumors with CDK4 amplification or overexpression.

CDK4 CN gains and CDKN2A deletions are frequently found in ACC (Assie et al., 2014, Lippert et al., 2018, Ross et al., 2014, De Martino et al., 2013). With regard to CDK4 and/or CDK6 overexpression, palbociclib was investigated in ACC cell lines by two recent studies observing a decrease of cell viability (Fiorentini et al., 2018, Hadjadj et al., 2017).

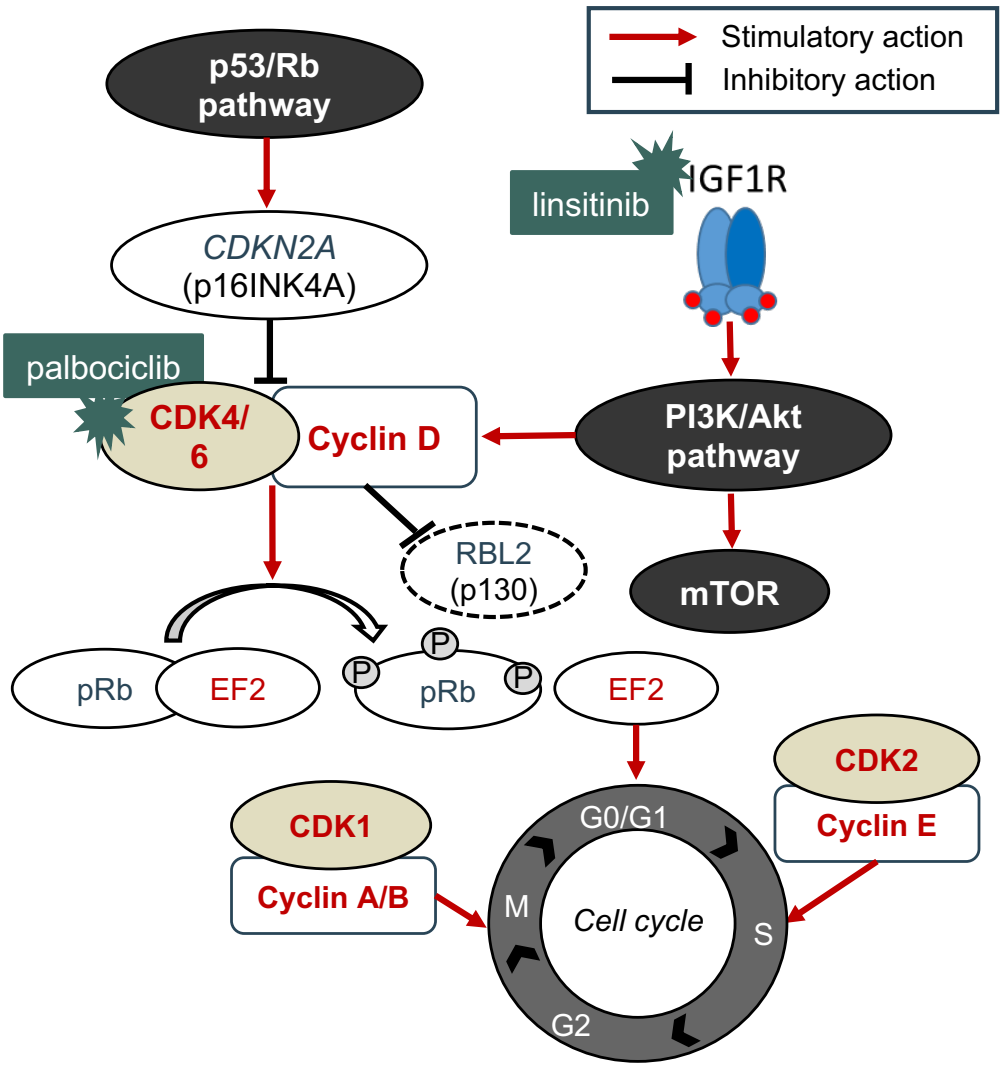


Figure 2.2: Schematic presentation of the CDK4 pathway including complexes of cyclin-dependent kinases and cyclins, regulatory factors with influence on cell cycle progression and available inhibitors. Activating elements are shown in red, suppressing elements in blue. This figure was modified according to Liang et al., 2020.

2.3.5. Cell biological methods

The effect of inhibition of selected drug target candidate CDK4 was investigated *in vitro* in ACC cell lines. Cell culture work was performed under low-germ conditions at a sterile work bench (Thermo Fisher Scientific) to avoid contamination. All material were sanitized by 70% ethanol. Culture medium and DPBS (Dulbecco's phosphate buffered saline) were pre-warmed in a 37 °C water bath before use.

2.3.5.1. Cell lines

Different cancer cell lines were used for *in vitro* experiments as listed in table 2.6.

Table 2.6: Cell lines used during this thesis.

cell line	histological type	Supplier
NCI-H295R	adrenocortical carcinoma cells	ATCC
MUC1	adrenocortical carcinoma cells	C. Hantel, University of Munich[1]
Hek293	embryonal kidney cells	ATCC
Hela	cervical carcinoma cells	ATCC

[1]Hantel et al., 2016
ATCC = American type culture collection.

2.3.5.2. Cultivation of ACC cells

Cells were cultivated in incubators at 37 °C, 95% relative humidity and 5% CO_2 and grown in T-75 flasks with 15 ml culture medium. For NCI-H295R cells, DMEM (Dulbecco's modified Eagle medium)/F12 (1:1) (Gibco) was supplemented with Nu-Serum (2.5%) (Corning) and insulin-transferrin-selenium (Gibco). MUC1 cells were cultured in Advanced DMEM/F12 (Gibco) supplemented with 10% FCS (fetal calf serum) and penicillin/streptomycin, while Hek-293AD and HeLa cells were cultured in DMEM/F12 (1:1) supplemented with 10% FCS. Cells growth was controlled by microscopy up to three times per week to assess morphology and cell density.

2.3.5.3. Passaging and seeding

Cells were split while being in log-phase of growth at 70-80% confluency as cell to cell contact at high cell densities in adherent cell lines can lead to contact inhibition as well as morphological changes and dedifferentiation of the cells.

After removing the culture medium from the flask, cells were washed once with 10 ml DPBS and incubated with 2 ml trypsin/EDTA (ethylenediamine tetraacetic acid) for 1 min to initiate cell detachment. About 2 min after trypsin/EDTA was taken off cells were completely detached and ready for resuspension in 10 ml fresh medium. NCI-H295R cells were passaged at a ratio of 1:2, MUC1 cells at a ratio of 1:3 and HeLa and Hek293 cells at a ratio of 1:10. Fresh culture medium was added accordingly to an end volume of 15 ml.

To achieve a certain number of cells for particular experiments, 10 µl of trypan blue dye was added to 10 µl of cell suspension. Viable cells remained unstained while non-viable cells were blued. The concentration of viable cells determined by the automated counting chamber was diluted appropriately with culture medium for each experiment and seeded in a suitable plate. NCI-H295R cells were plated at an amount of $1.4*10^5$ in 500 µl/well for a 24-well plate and at an amount of $2.5*10^4$ in 100 µl/well for a 96-well plate. MUC1 cells were plated at an amount of $5.6*10^4$ in 500 µl/well for a 24-well plate and at an amount of $1*10^4$ in 100 µl/well for a 96-well plate.

2.3.5.4. Transfection with small interfering RNA

CDK4 siRNA (small interfering RNA) was administered to ACC cells to achieve downregulation of *CDK4*. siRNA interferes with the expression of the gene of interest by hybridization with the complementary mRNA sequence leading to the degradation of the double-stranded mRNA. Additionally, targeting a pool of four mRNA regions allowed to improve effectiveness and specificity of silencing.

Cells were seeded out one day prior to transfection with eight technical replicates per condition in 96-well plates for WST1 (water soluble tetrazolium 1) analysis and 24-well plates for RNA and protein analysis. A 1 mM stock solution was prepared by diluting 10 nmol SMARTpool CDK4 siRNA (L-003238-00-0005, Dharmacon) in 10 µl nuclease-free water. To obtain the working solution of 1 µM, the stock solution was further diluted in serum-free growth medium. A non-coding siRNA control pool (D-001910-10-20) was used as negative control. Cells were washed with DPBS before 100 µl or 500 µl of 1 µM siRNA working solution was added to a 96-well or 24-well plate, respectively. After incubation for 96 h, cells were further processed.

2.3.5.5. Drug treatment

To investigate the potential and effect of different drug inhibitors on ACC, cells were treated with increasing drug concentrations of CDK4/6 inhibitor palbociclib (Pfizer), IGF1R/IR inhibitor linsitinib (Pfizer) and a combination of both. Cells were seeded out in 96-well plates with eight technical replicates per condition and 24-well plates for RNA and protein analysis. A primary stock concentration of 5 mM was achieved by addition of DMSO to palbociclib which was further diluted with DMSO to a secondary stock of the 310-fold concentration of the desired final working concentrations of 0.5 µM, 1 µM, 2 µM, 4 µM, 8 µM and 16 µM by addition of culture medium. Similarly, DMSO was added to linsitinib for a primary stock concentration of 5 mM and diluted with DMSO to a secondary stock of the 1000-fold concentration of the desired final working concentrations of 0.125 µM, 0.25 µM, 0.5 µM, 1 µM, 2 µM and 4 µM by addition of culture medium. To combine both drugs 4 µM of palbociclib and 0.25 µM linsitinib were diluted in appropriate amounts of culture medium. DMSO concentration remained under 0.45% for every drug application. Drug-containing medium was replaced every other day.

2.3.5.6. Cell viability test by WST1 analysis

A WST1 assay (Sigma-Aldrich) was performed to assess changes of cell viability. Cells were incubated with WST1 solution for 2 h according to manufacturer's instructions. Viable cells with an undamaged respiratory chain of the mitochondria and intact succinate-tetrazolium reductase system cleave tetrazolium salts to a dark red formazan which can be photometrically measured by a spectrophotometer with the absorbance correlating with the number of viable cells.

2.3.6. Biochemical methods

WBs (western blots) were used to assess protein expression of the CDK4/6 pathway in ACC cell lines as well as to demonstrate changes on the protein expression of the CDK4/6 pathway after treatment of ACC cells.

2.3.6.1. Preparation of whole protein cell lysates

Cell lysates were prepared for further investigation of the proteins of interest in WB. Culture medium was discarded to allow cell lysis with 250 µl of RIPA buffer (Sigma)

mixed with 1x protease and phosphatase inhibitor cocktails in a 6-well plate. Following an incubation for 10 min at 4 °C the cell lysate was then centrifuged in a 1.5 ml reaction tube at 14000 rpm for another 10 min at 4 °C. The supernatant was then transferred to a novel 1.5 ml reaction tube and stored at -20 °C. The amount of RIPA buffer was appropriately modified for cells grown in a 24-well plate.

2.3.6.2. Protein quantification by bicinchoninic acid assay

Protein concentration in cell lysates was quantified by a BCA (bicinchoninic acid) kit (Sigma Aldrich) according to manufacturer's instructions. After an incubation for 30 min at 37 °C, the absorbance was measured with a photometer and correlated with a BCA-standard to obtain protein concentration of the investigated samples.

2.3.6.3. Sodium dodecyl sulfate polyacrylamide gel electrophoresis

SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) allowed the separation of macromolecules dependent on relative molecular mass and was performed using a discontinuous polyacrylamide gel as support medium and 5x SDS loading buffer which was mixed in a 1:5 ratio with cell lysates to mask charges of the proteins. The sample was then denaturized for 5 min at 98 °C and equal amounts of protein were loaded on a 4-20% polyacrylamide gradient gel (BioRad) together with 3.5 µl of page ruler prestained protein ladder (Thermo Fisher) as size standard. Polypeptides were then separated in SDS running buffer at 80 V for 20 min and for 150 V for 45 min. The composition of the buffers used for gel electrophoresis can be found below.

2.3.6.4. Western blot

Specific polypeptides were identified and quantified by WB. The nitrocellulose membrane, gel and Whatman filters were equilibrated in transfer buffer and electrophoretic transfer to the membrane was performed through tank-blot at 100 V for 1 h at 4 °C. After blocking of unspecific binding sites in 5% milk-TBS-T (tris(hydroxymethyl)aminomethane-buffered saline with Tween20) for 1 h at RT, the membrane was probed with antibodies for the gene of interest diluted to appropriate concentrations in 5% milk-TBS-T overnight at 4 °C on a shaker. The utilized antibodies and dilutions as applied for this thesis are listed in table 2.7. Following three washing

steps of 10 min with TBS-T, signal detection was achieved by incubation with respective HRP labelled secondary antibodies diluted 1:10000 in TBS-T for 1 h at RT. The membrane was then washed for another three times as described above and incubated with Amersham ECL Prime reagent (GE Healthcare) for 1 min according to manufacturer's instructions leading to visualization of the protein-antibody complex by enhanced chemiluminescence. The signal was captured on X-ray film which was then developed and fixed manually. Scanning allowed the quantification of individual bands with the FIJI software (version 2.0.0 (Schindelin et al., 2012)). The composition of the buffers used for blotting can be found below.

Table 2.7: Primary and secondary antibodies utilized for WB.

antigen	**clone or number**	**host**	**supplier**	**application and dilution**
primary antibodies				
α-tubulin	DM1A	mouse	Sigma-Aldrich	WB (1:20000)
CDK4	EPR4513-32-7	rabbit	Abcam	WB (1:1000)
$p16^{INK4A}$	G175-405	mouse	BD Pharmingen	WB (1:500)
RB	4H1	mouse	Cell Signaling	WB (1:2000)
p130/RBL2	D9T7M	rabbit	Cell Signaling	WB (1:1000)
secondary antibodies				
goat-α-mouse-HRP	115-035-003	goat	Jackson ImmunoResearch	WB (1:10000)
goat-α-rabbit-HRP	111-035-144	goat	Jackson ImmunoResearch	WB (1:10000)

Abbreviations: WB = western blot; CDK = cyclin-dependent kinase, CDKN2A = cyclin-dependent kinase inhibitor 2A; RB = retinoblastoma; RBL2 = retinoblastoma-like 2; HRP = horseradish peroxidase.

Buffers utilized for SDS-PAGE and WB:

- **Laemmli sample buffer (5x)**
  0,3 M Tris hydrochloric acid (pH 6.8)
  10 mM dithiothreitol
  5% SDS
  50% glycerol
  0,5% bromphenol blue
- **SDS Running buffer**
  25 mM Tris
  192 mM glycine
  0.1% SDS
- **Transfer buffer**
  25 mM Tris
  0.2 mM glycine
  10% methanol
- **TBS-T**
  20 mM Tris
  200 mM sodium chloride
  0.01% Tween-20
- **Stripping buffer**
  25 mM glycine
  1% SDS
  pH 2

2.3.6.5. Re-probing of nitrocellulose membranes

For re-use of a membrane, removal of primary and secondary antibodies was achieved by incubation with stripping buffer twice for 25 min at RT. After three washing steps, membranes were blocked and re-probed with a novel antibody as described above.

2.3.7. Statistical analysis

A Fisher's exact or Chi-square test was used to investigate dichotomic variables, while a two-sided t-test or non-parametric Mann-Whitney test was used to compare two groups of continuous variables as appropriate. A non-parametric Kruskal-Wallis test followed by Bonferroni post-hoc test was used for comparison among several groups for non-normal distributed variables. Correlations and 95%CI between different parameters were evaluated by linear regression analysis. OS was defined as the time from the date of primary surgery to specific death caused by ACC or last follow-up, while PFS was defined as the time from the date of complete tumor resection to the first radiological evidence of disease relapse or disease-related death. TTP during therapy was defined as the time from the date of first drug administration to the first radiological evidence of any kind of disease progression or relapse or death. Survival curves were obtained by Kaplan-Meier estimates and the differences between two or more curves were investigated by the log-rank (Mantel-Cox) test. A multivariate regression analysis was performed by Cox proportional hazard regression model to identify those factors that might independently influence survival. Statistical analyses were performed with GraphPad Prism software 6.0 (GraphPad Software Inc., La Jolla, CA, USA) and p-values below 0.05 were considered statistically significant.

3. Results

3.1. Validation of FFPE mRNA extraction

To demonstrate the feasibility of good quality RNA isolation from FFPE tissue, RT2 Profiler PCR Array was performed using RNA obtained from five matched samples from fresh frozen material and FFPE specimen. Results of the RT2 Profiler correlated with high significance between the two material types ($p < 0.0001$, R = 0.80) (figure 3.1).

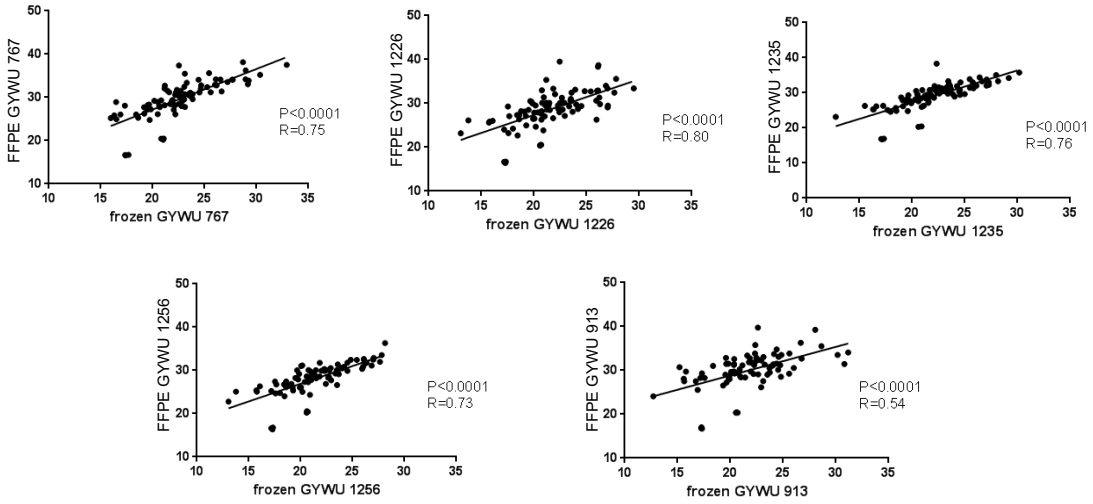


Figure 3.1: Comparison of gene expression of 84 known cancer drug target genes in FFPE tissue and frozen tissue in 5 randomly selected ACC samples (RT-qPCR profile, for details see Material and Methods)**.**

3.2. Gene expression profile

In 40/107 ACC cases with good quality RNA isolated from FFPE, gene expression of 84 cancer drug target genes was assessed by RT2 Profiler PCR Array. A relative overexpression with a FC ≥ 2.0 in at least 25% of cases was detected in 16 genes whereas 54 genes showed a FC < 2 in at least 25% of cases. Overall, low expression (FC ≤ -2.0) was detected in 80 genes in at least one sample while an overexpression (FC ≥ 2.0) was detected in 74 genes. Of those, 37 genes presented very high expression (FC ≥ 5.0) in at least one sample. No case of overexpression occurred in 10 genes. The percentage of samples with high or very high expression of each investigated gene is shown in figure 3.2.

The six most frequently upregulated genes were *TOP2A* (100% of cases, median FC 16.5, range: 2.0 to 126.7), *IGF2* (95% of cases, median FC 52.9, range: -58.6 to 532.9), *CDK1* (80% of cases, median FC 6.7, range: -1.3 to 27.4), *CDK4* (62% of cases, median FC 2.6, range: -1.5 to 15.0), *PLK4* (60% of cases, median FC 2.8, range: -1.6 to 36.0) and *PLK1* (52% of cases, median FC 2.3, range: -2.7 to 33.9).

Furthermore, several gene families were observed to be overexpressed as an entity with at least 50% or more samples upregulated including AURK (aurora kinase) (62% of cases with at least one member overexpressed), CDK (95% of cases) and PLK (75% of cases) (figure 3.2).

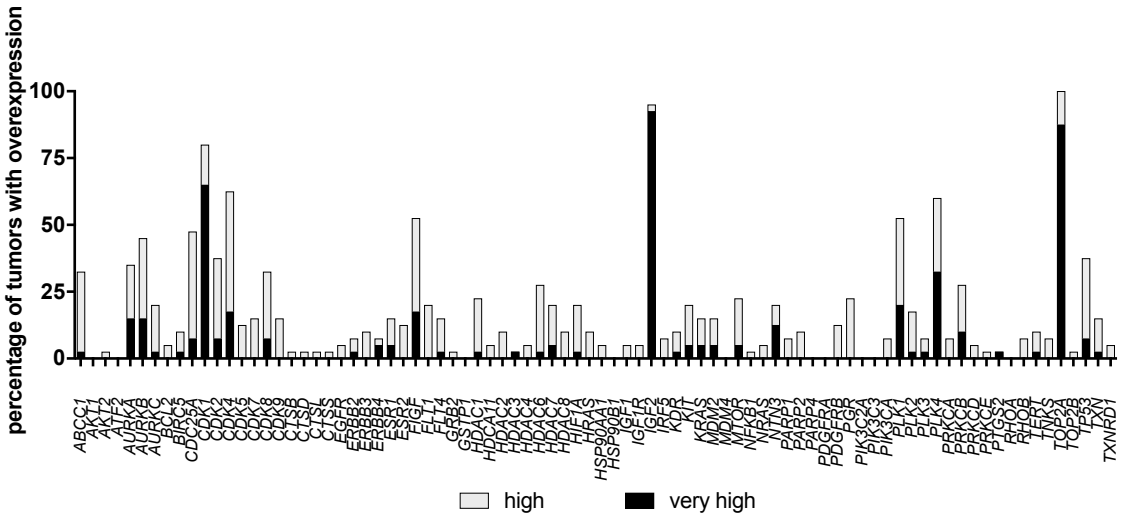


Figure 3.2: Percentage of samples with relative overexpression for the 84 cancer drug target genes. A fold change of ≥ 2.0 was defined as high expression and a fold change ≥ 5.0 was defined as very high expression. This figure was modified according to Liang et al., 2020.

Notably, *IGF2* presented a strong overexpression, whereas expression of both *IGF1* and *IGF1R* was moderate and downregulated in 60% and 45% of cases, respectively (median FC -2.9, range: -67.8 to 3.0, and median FC -1.7, range: -25.5 to 3.0, respectively). In addition, 37.5% of samples were observed with both high *CDK4* and normal or high *IGF1R* mRNA expression. However, no specific association was found between *CDK4* and *IGF2*, *IGF1* or *IGF1R* expression (figure 3.3).

Similarly, while overexpression of *TOP2A* was observed in the entire cohort, *TOP2B* was rather lowly expressed (median FC -2.2, range -11.9 to 2.5).

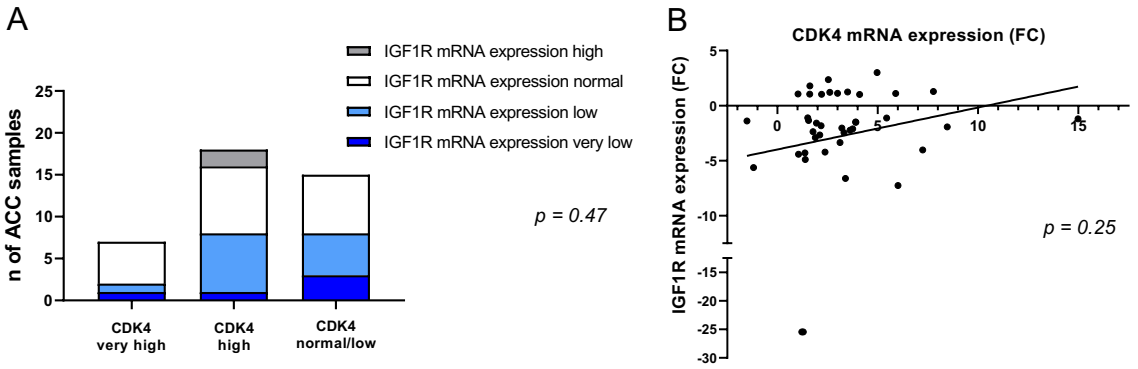


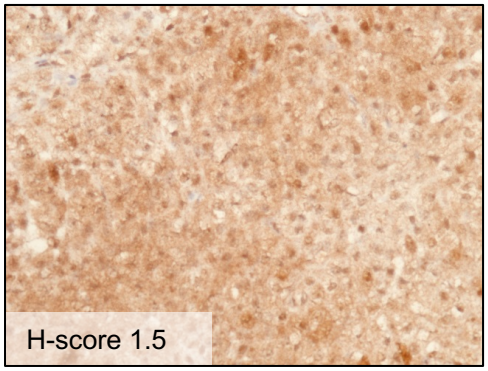
Figure 3.3: Association between *CDK4* and *IGF1R* gene expression in the mRNA cohort.
A) Distribution of ACC samples with different *CDK4* and *IGF1R* gene expression levels. Statistical analysis by Chi-square test.
B) Relationship between CDK4 and IGF1R mRNA fold change (p = 0.25). The regression line is shown. Statistical analysis by linear regression.
This figure was modified according to Liang et al., 2020.

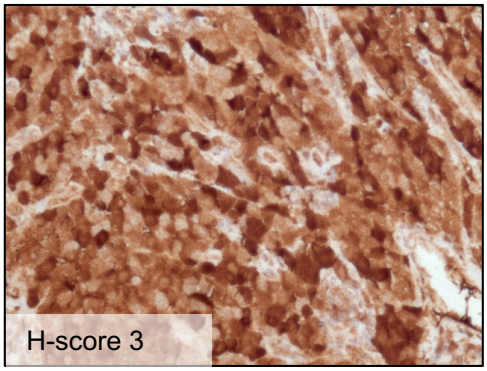
3.3. Protein expression by IHC in four candidate genes, relationship of expression in ACC, ACA and NAG and correlation with mRNA data.

As described previously (see section 2.3.3), four of the six most frequently overexpressed genes (*CDK1*, *CDK4*, *PLK1* and *TOP2A*) were selected for further investigation and further assessment by validation of their expression on protein level by IHC.

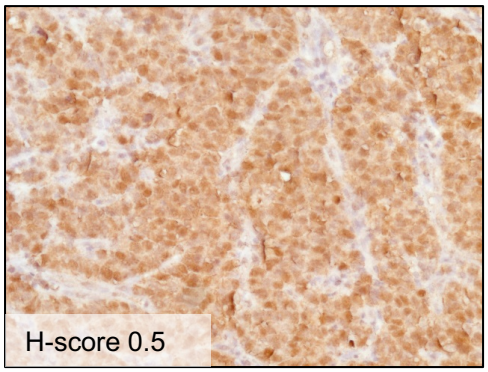
Two representative examples of stained ACC samples are shown for each protein in figure 3.4 A-D.

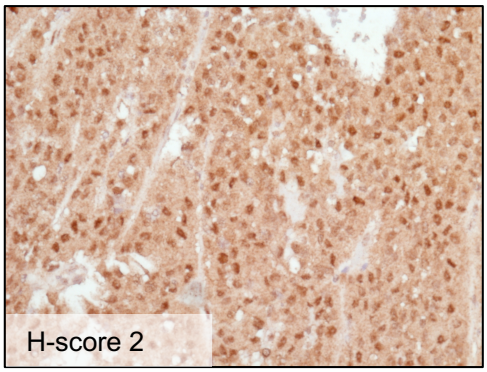
A


H-score 1.5

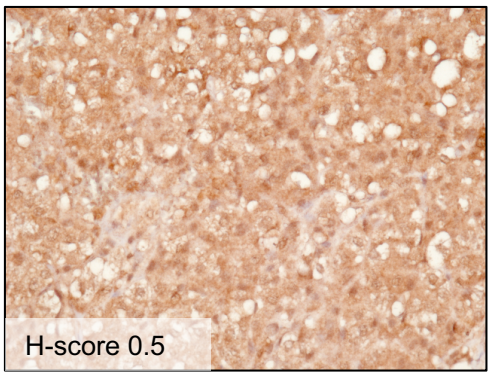

H-score 3

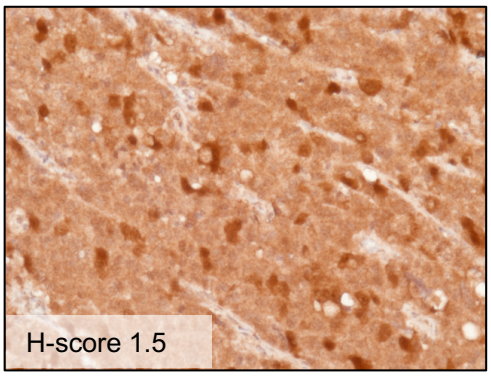
B


H-score 0.5

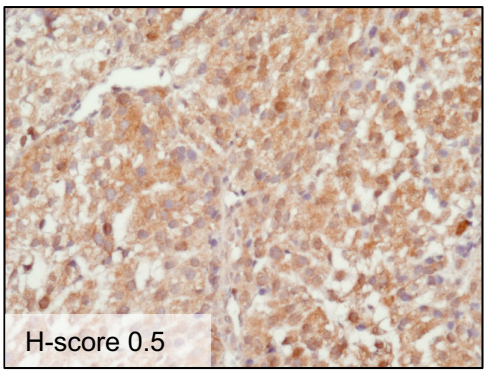

H-score 2

C


H-score 0.5


H-score 1.5

D


H-score 0.5

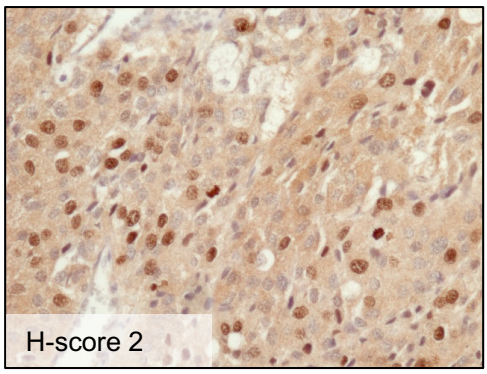

H-score 2

Figure 3.4: Examples of immunostaining for four selected candidate genes in ACC samples. Magnification 1x20.
A) Examples of CDK1 immunostaining in two samples, one with intermediate positive nuclear staining (H-score 1.5) and one with strongly positive nuclear staining (H-score 3).
B) Examples of CDK4 immunostaining in two samples, one with weak positive nuclear staining (H-score 0.5) and one with moderate positive nuclear staining (H-score 2).
C) Examples of PLK1 immunostaining in two samples, one with weak positive nuclear staining (H-score 0.5) and one with intermediate positive nuclear staining (H-score 1.5).
D) Examples of TOP2A immunostaining in two samples, one with weak positive nuclear staining (H-score 0.5) and one with moderate positive nuclear staining (H-score 2).
Figure 3.4 B was modified according to Liang et al., 2020.

Overall, the mRNA expression data were correlated with the IHC results (n = 40, mRNA cohort). Specifically, *CDK1*, *CDK4* and *PLK1* mRNA fold change significantly correlated with correspondent nuclear H-score ($p < 0.001$, R = 0.66; $p = 0.0005$, R = 0.52; p = 0.045, R = 0.32) while no significant relationship was observed between *TOP2A* mRNA expression and protein expression (figure 3.5 A-D).

Furthermore, the IHC results of the original ACC cohort (n = 104) were compared with ACA and NAG tissue to investigate differences of expression. CDK1 nuclear H-score was significantly higher in ACC and ACA than in NAG ($p < 0.01$ and $p < 0.05$, $p = 0.0094$ per trend) (figure 3.5 A), while CDK4 nuclear H-score showed a significant upregulation in ACC in respect to ACA and NAG (both $p < 0.01$, $p < 0.0002$ per trend) with no difference between ACA and NAG (figure 3.5 B). PLK1 protein expression differed not significantly in the three adrenocortical tissue entities (figure 3.5 C), similar to TOP2A protein expression though TOP2A nuclear H-score presented a significant decreasing trend in ACC, ACA and NAG ($p = 0.0249$) (figure 3.5 D).

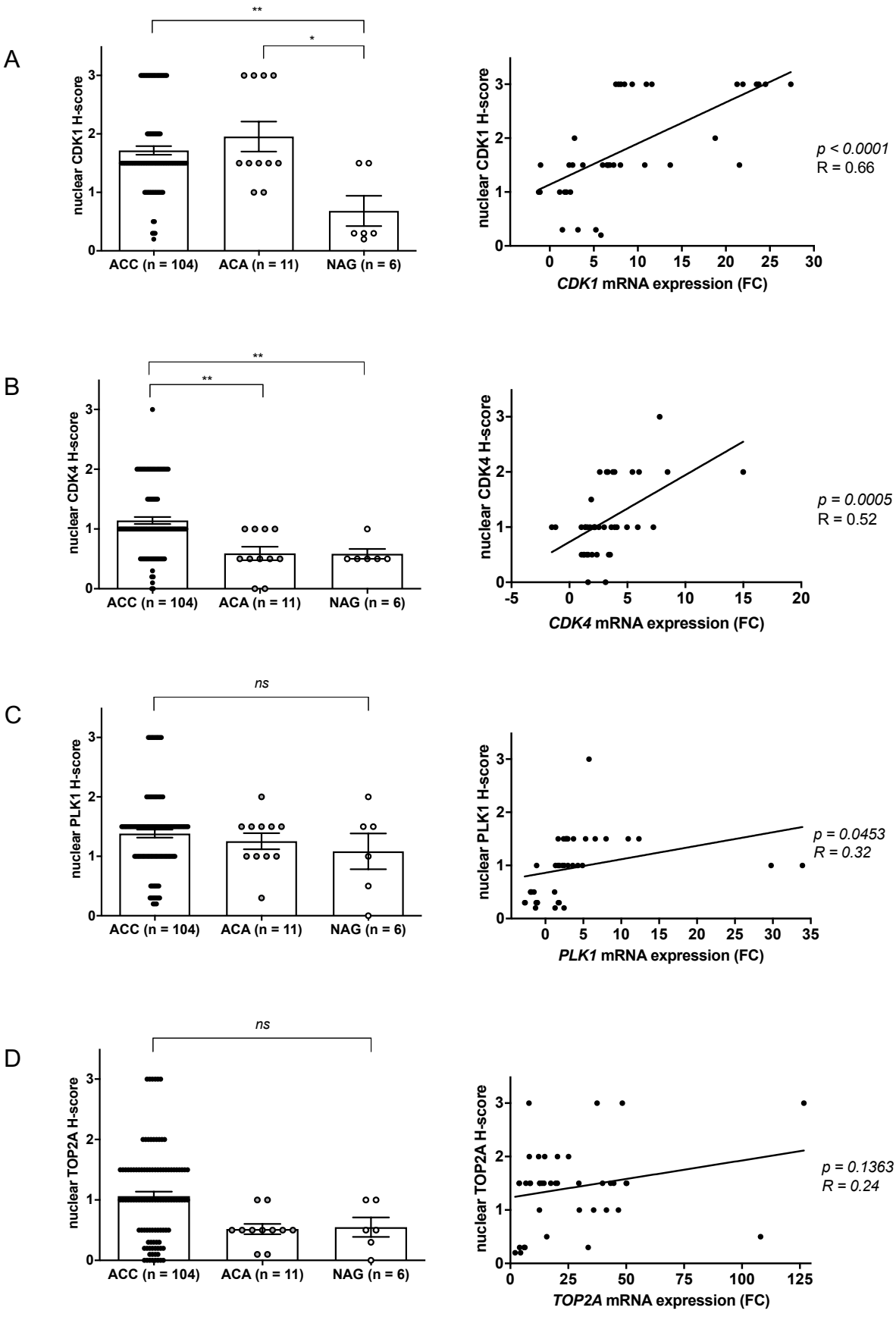

A
**
*
nuclear CDK1 H-score
ACC (n = 104)
ACA (n = 11)
NAG (n = 6)
nuclear CDK1 H-score
CDK1 mRNA expression (FC)
p < 0.0001
R = 0.66
B
**
**
nuclear CDK4 H-score
ACC (n = 104)
ACA (n = 11)
NAG (n = 6)
nuclear CDK4 H-score
CDK4 mRNA expression (FC)
p = 0.0005
R = 0.52
C
ns
nuclear PLK1 H-score
ACC (n = 104)
ACA (n = 11)
NAG (n = 6)
nuclear PLK1 H-score
PLK1 mRNA expression (FC)
p = 0.0453
R = 0.32
D
ns
nuclear TOP2A H-score
ACC (n = 104)
ACA (n = 11)
NAG (n = 6)
nuclear TOP2A H-score
TOP2A mRNA expression (FC)
p = 0.1363
R = 0.24

Figure 3.5: Evaluation of protein expression in four selected candidate genes in ACC.
Comparison of nuclear protein expression evaluated by IHC in ACC samples (n = 104), ACA (n = 11) and NAG (n = 6) (left). Bars represent median and interquartile range. Statistical analysis by Kruskal-Wallis test. Relationship between protein expression and mRNA expression evaluated by fold change (right). The regression line is shown. Statistical analysis by Pearson *r* correlation test.
A) Analyses of CDK1 protein expression in the three adrenocortical entities (*p = 0.0094* per trend) as well as in relationship with *CDK1* mRNA expression. *p < 0.05, **p < 0.01
B) Analyses of CDK4 protein expression in the three adrenocortical entities (*p = 0.0002* per trend) as well as in relationship with *CDK4* mRNA expression. **p < 0.01
C) Analyses of PLK1 protein expression in the three adrenocortical entities (*p = 0.6970* per trend) as well as in relationship with *PLK1* mRNA expression. ns = p not significant
D) Analyses of TOP2A protein expression in the three adrenocortical entities (*p = 0.0249* per trend) as well as in relationship with *TOP2A* mRNA expression. ns = p not significant
Figure 3.5 B was modified according to Liang et al., 2020.

3.4. Relationship with clinical data

We investigated the relationship between RNA and protein data and available clinical data such as ENSAT tumor stage, Ki67 proliferation index as well as OS and PFS. Considering the mRNA cohort (n = 40), a weak inverse relationship was observed between ENSAT tumor stage and IGF1R expression (*p = 0.0355*, data not shown) as well as a positive relationship regarding *KRAS* mRNA expression (*p = 0.022*, data not shown). A significant positive correlation was detected between Ki67 proliferation index and mRNA expression of *AURKB*, *CDC25A* (cell division cycle 25A), *CDK1*, *CDK2*, *FLT1* (fms-related tyrosine kinase 1), *HDAC2* (histone deacetylase 2), *MTOR*, *PARP1* (poly(ADP-ribose) polymerase 1), *PDGFRB* (platelet derived growth factor receptor beta) and *TOP2A*. An additional inverse correlation was presented by *PRKCA* (protein kinase C alpha) (data not shown). Two investigated candidate genes, CDK1 and TOP2A, retained a significant correlation with Ki67 proliferation index on protein level in the entire cohort (n = 104, data not shown).

Regarding the clinical outcome in the mRNA cohort (n = 40), high *CDK1* and *HIF1A* mRNA expression significantly correlated with worse OS and PFS, whereas *ERBB4* and *TXNRD1* mRNA expression were positively related with better OS and PFS. Considering solely PFS, patients with high *PARP1* mRNA expression showed worse and patients with high *CDK8*, *PRKCA* and *HDAC* mRNA expression showed better survival rates.

Furthermore, OS was positively linked to high *ABCC1* mRNA expression (data not shown).

In the entire cohort (n = 104) high CDK4 nuclear H-score was associated with longer PFS in respect to patients with low CDK4 protein expression (median = 24 *vs* 9 months, *p = 0.0122*, HR = 0.56, 95%CI = 0.36-0.88) (figure 3.6 A). Generally, PFS was also significantly related to ENSAT tumor stage (stage 1-2 *vs* 3-4, *p = 0.001*, HR = 2.25, 95%CI = 1.39-3.64), Ki67 proliferation index (cut-off 15%, *p < 0.001*, HR = 3.53, 95%CI = 2.11-5.89), and resection status (R0-X *vs* R1-2, *p < 0.001*, HR = 6.49, 95%CI = 2.5-16.8) as expected. A multivariate analysis including these parameters showed a remaining significant association between CDK4 expression and PFS (*p = 0.044*, HR = 0.45, 95%CI = 0.20-0.98). This observation was also true considering the subset of patients treated with platinum compounds (n = 53) separately when high CDK4 protein expression at tumor level was significantly associated with longer TTP (median 6 *vs* 4 months, *p = 0.0156*, HR = 3.1, 95%CI=1.4-6.7) (figure 3.6 B). No significant relationship was observed between CDK4 gene expression and survival in previous ACC cohorts (n = 33 (Giordano et al., 2009), n = 45 (Assie et al., 2014), n = 77 (Zheng et al., 2016), data not shown).

A significant relationship between TOP2A protein expression and TTP in EDP regimen treated patient cohort (n = 53, *p = 0.5608*) was not found in this study (figure 3.7).

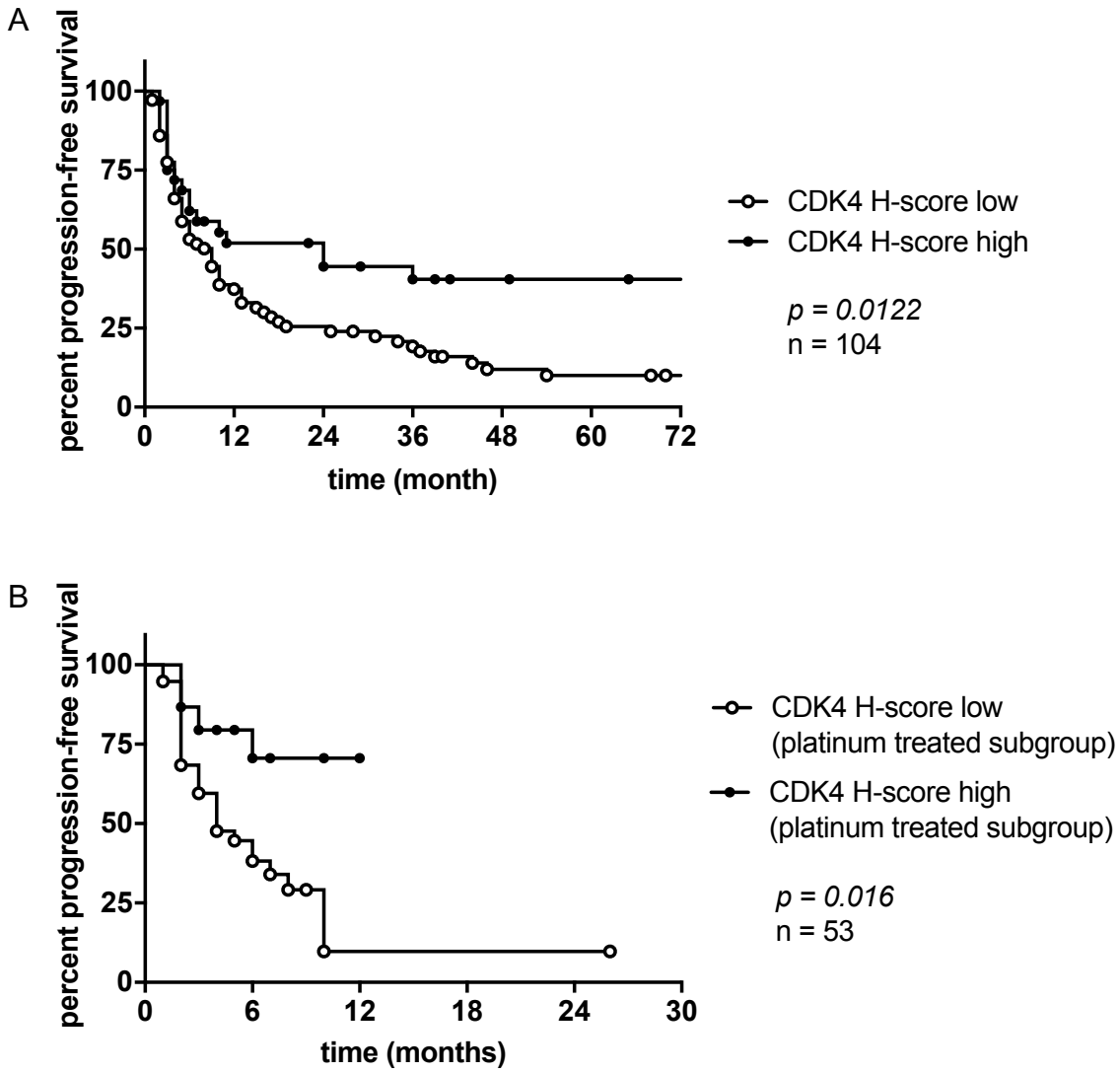


Figure 3.6: Association of survival rates and CDK4 nuclear protein expression.
A) Comparison of PFS curves regarding low CDK4 expression defined as H-score ≤ 1 (n = 72) and high expression defined as H-score > 1 (n = 32). Statistical analysis by log-rank test.
B) Evaluation of TTP in platinum-treated ACC subgroup (n = 53) comparing low expression (n = 38) and high expression (n = 15) as defined above. Statistical analysis by log-rank test.
This figure was modified according to Liang et al., 2020.

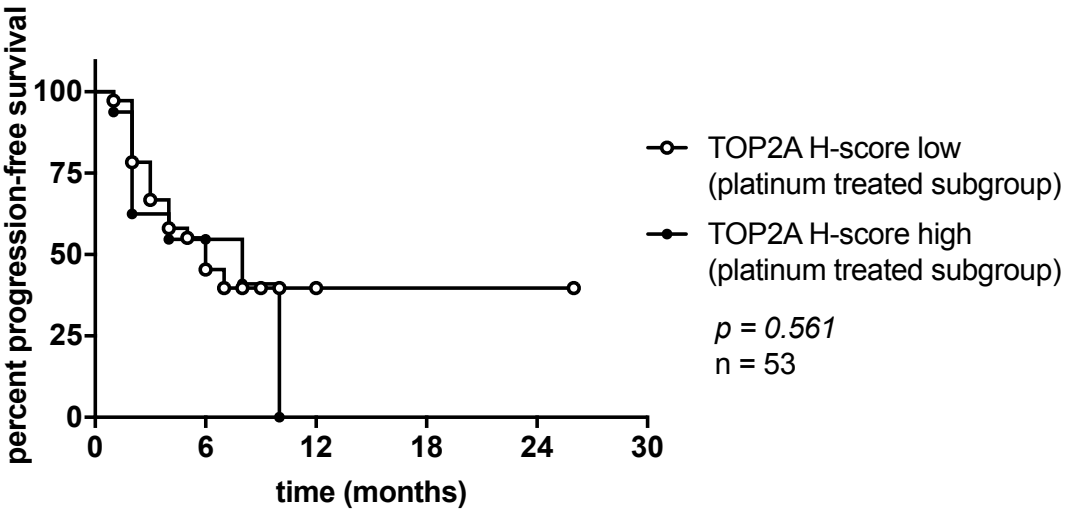


Figure 3.7: Association of TTP and TOP2A nuclear protein expression. Evaluation of TTP in platinum-treated ACC subgroup (n = 53) comparing low TOP2A expression defined as H-score ≤ 1 (n = 37) and high expression defined as H-score > 1 (n = 16). Statistical analysis by log-rank test.

3.5. Selection of best drug target candidate CDK4

On the basis of the criteria mentioned above (see section 2.3.3), CDK4 was selected as the most promising drug target candidate for further examination in ACC cell lines. *CDK4* CN gains as well as losses of its regulator *CDKN2A* are frequently reported alterations in ACC (Assie et al., 2014, Zheng et al., 2016, De Martino et al., 2013). The entire present ACC cohort was previously analyzed by targeted next generation sequencing (Lippert et al., 2018) and *CDK4* CN gains were presented in 43% of cases. Comparison of CDK4 mRNA expression and CDK4 CN status in the mRNA subcohort (n = 40) revealed a significantly higher mRNA expression in ACC with *CDK4* CN gain (n = 16) than ACC with normal *CDK4* CN status (*p = 0.0085*, figure 3.8 A). However, only a positive trend was found between *CDK4* CN status and CDK4 protein expression in the entire cohort (n = 104, *p = 0.2285*, data not shown).

A stratification of the cohort by low and high CDK4 nuclear H-score is listed in table 3. Similarly, available transcriptome data sets (Giordano et al., 2009) showed a significantly higher *CDK4* expression in ACC in comparison to both ACA and NAG (figure 3.8 B).

Table 3: Clinical and histopathological data in the entire cohort of patients with ACC (n = 104) stratified by CDK4 protein expression (Liang et al., 2020)**.**

	low CDK4 expression H-score ≤ 1	**high CDK4 expression** H-score > 1	**p value**
n	**72**	**32**	-
Sex (F/M)	43/29	16/16	ns
Baseline			
Age – yrs (median, range)	46 (18-87)	51 (25-81)	
< 50 years – *n (%)*	40 (55.6)	14 (43.8)	ns
≥ 50 years – *n (%)*	32 (44.4)	18 (56.2)	
Steroid secretion – *n* available	55	23	
Cortisol – *n (%)*	14 (19.4)	9 (28.1)	
Other single steroids (androgens, mineralocorticoids, or estrogens) – *n (%)*	7 (9.7)	2 (6.3)	ns
Mixed steroids – *n (%)*	14 (19.4)	7 (21.9)	
Inactive – *n (%)*	20 (27.8)	5 (15.6)	
Tumor localization			
Primary tumor – *n (%)*	57 (79.2)	30 (93.8)	*0.08*
Local recurrences – *n (%)*	7 (9.7)	1 (3.1)	
Metastases – *n (%)*	8 (11.1)	1 (3.1)	
ENSAT tumor stage			
I-II – *n (%)*	38 (52.8)	17 (53.1)	ns
III – *n (%)*	16 (22.2)	11 (34.4)	
IV – *n (%)*	18 (25.0)	4 (12.5)	
Resection status – *n* available	70	31	
R0 – *n (%)*	46 (63.9)	26 (81.3)	
RX – *n (%)*	12 (16.7)	4 (12.5)	ns
R1 – *n (%)*	5 (6.9)	0 (0)	
R2 – *n (%)*	7 (9.7)	1 (3.1)	
Ki67 index – median (range)	15 (2-90)	10 (1-80)	ns
Therapeutic approaches			
Additional surgeries – *n (%)*	30 (41.7)	8 (25.0)	ns
Radiotherapy (tumor bed or metastases) – *n (%)*	25 (34.7)	7 (21.9)	ns
Mitotane			
Adjuvant setting – *n (%)*	28 (38.9)	10 (31.3)	ns
Palliative setting – *n (%)*	32 (44.4)	6 (18.8)	*0.01*
Cytotoxic chemotherapies			
None – *n (%)*	27 (37.5)	14 (43.7)	ns
Platinum-based regimen – *n (%)*	38 (52.8)	15 (46.9)	
Streptozotocin – *n (%)*	31 (43.1)	12 (37.5)	
Gemcitabin plus capecitabin – *n (%)*	27 (37.5)	10 (31.3)	
Iodmetomidate – *n (%)*	4 (5.6)	0 (0)	ns

Abbreviations: F = female; M = male; n = number of patients; R0 = complete resection; R1 = microscopic incomplete resection; R2 = macroscopic incomplete resection; RX = uncertain resection; yrs = years.

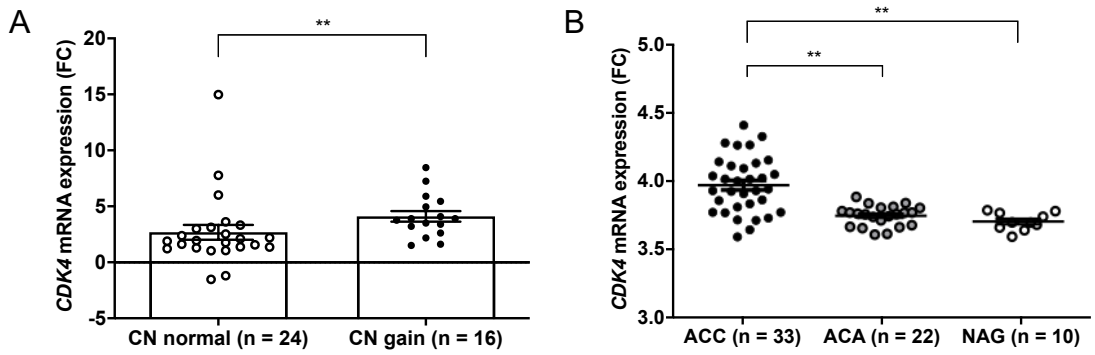


Figure 3.8: CDK4 as cancer drug target candidate.
A) Relationship between *CDK4* gene expression and CN status in the RNA cohort (n = 40) with *CDK4* mRNA FC in ACC with normal CN status (n = 23) or CDK4 CN gain with CN gain heterozygous (n = 13) or homozygous (n = 4) as published by (Lippert et al., 2018). Bars represent median and interquartile range. *p < 0.05. Statistical analysis by Kruskal-Wallis test followed by Bonferroni post-hoc test.
B) *CDK4* gene expression after quantile normalization and log transformation in ACC (n = 33), ACA (n = 22) and NAG (n = 10) (Giordano et al., 2009). Bars represent median and interquartile range. **p < 0.01. Statistical analysis by Kruskal-Wallis test.
This figure was modified according to Liang et al., 2020.

3.6. *In vitro* drug targeting of CDK4

To assess the potential of CDK4 as a drug target, the gene was investigated *in vitro* using the standard adrenocortical cancer cell line NCI-H295R and the newly established cell line MUC1.

3.6.1. Expression of CDK4 related genes and proteins in ACC cell lines

To determine the suitability of the utilized ACC cell lines and facilitate a potential application to a subcohort of ACC patients, characterization of the cell lines was performed on DNA and mRNA level and validated on protein level regarding CDK4-related genes (shown in figure 2.2). No CN variations in *CDK4* or *CDKN2A* were detected in neither NCI-H295R nor MUC1 cells whereas *RB* gene losses could be observed in the NCI-H295R but not in the MUC1 cell line (performed by J. Lippert). On mRNA level, *CDK4* was relatively high expressed in NCI-H295R cells (2.50 ± 1.01) and moderately expressed in MUC1 cells (0.81 ± 0.25) compared to two other *CDK4* expressing cell lines Hek293 and Hela cells as positive controls (figure 3.9 A). These findings were mirrored on protein level by WB analysis (figure 3.9 B). *CDKN2A* mRNA expression was similarly upregulated in both NCI-H295R (2.30 ± 1.19) and MUC1 (2.89 ± 0.53) cells,

whereas $p16^{INK4A}$ protein expression was notably lower in NCI-H295R cells than in MUC1 cells. No *RB1* mRNA expression was found in NCI-H295R cells while MUC1 cells presented low *RB1* expression levels, however RB protein expression was not detectable in neither of both cell lines. p130/RBL2 protein, an additional CDK4 related phosphorylation target previously reported to be expressed in NCI-H295R cells (Fiorentini et al., 2018), was investigated as surrogate and was observed to be consistently expressed in both cell lines. Low expression of *CDK1*, *CDK6* and *CCND1* mRNA were displayed by both investigated ACC cell lines (all < 0.4) (figure 3.9).

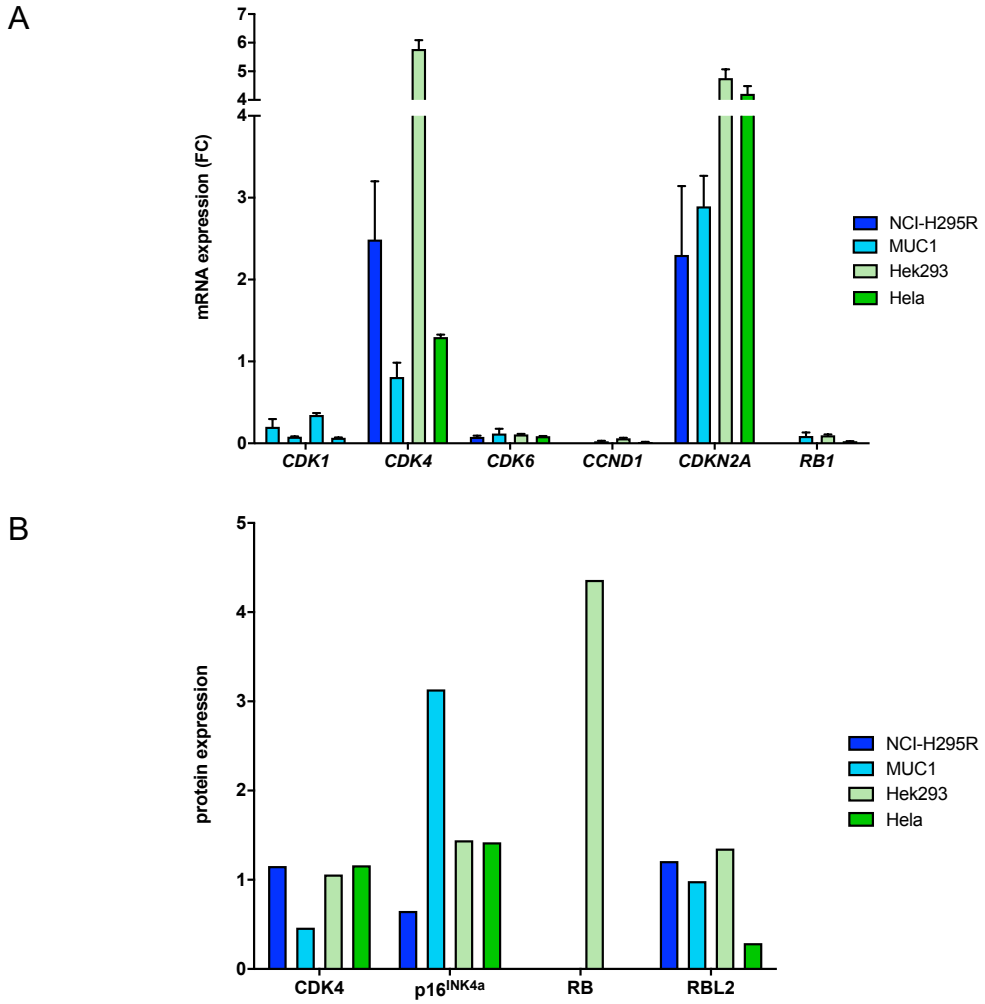


Figure 3.9: RNA and protein expression of CDK4-related factors in two ACC cell lines.
A) mRNA expression levels of *CDK1*, *CDK4*, *CDK6*, *CCND1*, *CDK2NA* and *RB1* in ACC cell lines NCI-H295R and MUC1 cells (triplicates by RT-qPCR). Hek293 and Hela cells were used as positive controls and β-actin was used as housekeeping gene as internal standard.
B) Quantitative WB analysis of CDK4, $p16^{INK4A}$, RB and p130/RBL2 for NCI-H295R and MUC1 cells as well as Hek293 and Hela cells. α-tubulin was used as the internal standard. Each bar of the histograms represents the mean of the ratio of protein of interest to α-tubulin signal (n = 1). This figure was modified according to Liang et al., 2020.

Targeted RT profiling of the two cell lines presented very high mRNA expression levels of *IGF2* (FC < 2000) and high levels of *IGF1R* (FC 4.1) along with low expression of *IGF1* (FC -32) in NCI-H295R cells. MUC1 cells showed overexpression of *IGF2* (FC < 20.0) and *IGF1R* (FC 2.8) and regular expression of *IGF1* (FC 1.2) (figure 3.10).

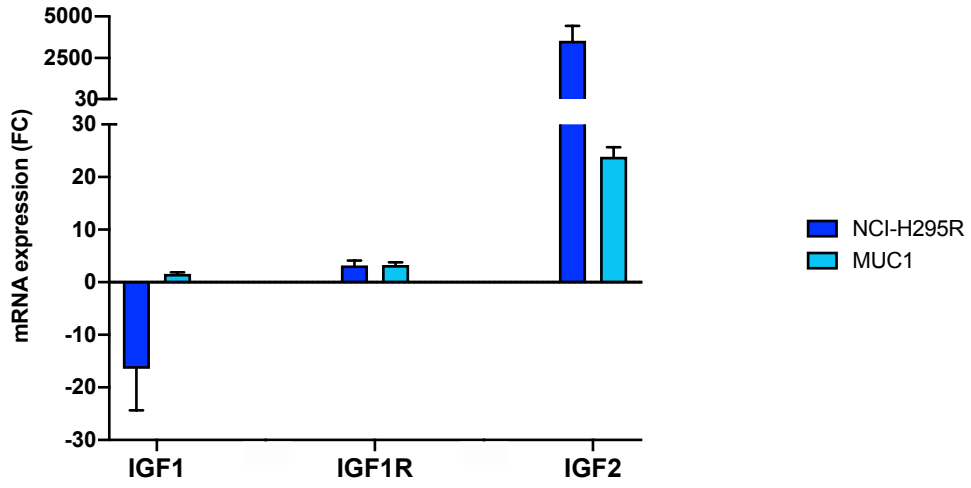


Figure 3.10: RNA and protein expression of the IGF family in two ACC cell lines. mRNA expression levels of *IGF1*, *IGF1R* and *IGF2* in ACC cell lines NCI-H295R and MUC1 cells. β-actin was used as housekeeping gene as internal standard. Experiments were conducted in triplicates. This figure was modified according to Liang et al., 2020.

3.6.2. CDK4 inhibition by siRNA

In order to assess the impact of *CDK4* inhibition on cell viability, CDK4 siRNA was introduced to NCI-H295R and MUC1 cells. Successful transfection was confirmed 72 h after treatment by RT-qPCR and WB. In comparison with mock transfected cells, significant reduction of CDK4 at both mRNA level (60% reduction, $p = 0.0175$ and 85% reduction, $p = 0.0022$ for NCI-H295R cells and MUC1 cells, respectively, figure 3.11 A) and protein level (80% reduction, $p = 0.0039$ and 95% reduction, $p = 0.0004$ for NCI-H295R cells and MUC1 cells, respectively, figure 3.11 B) was obtained.

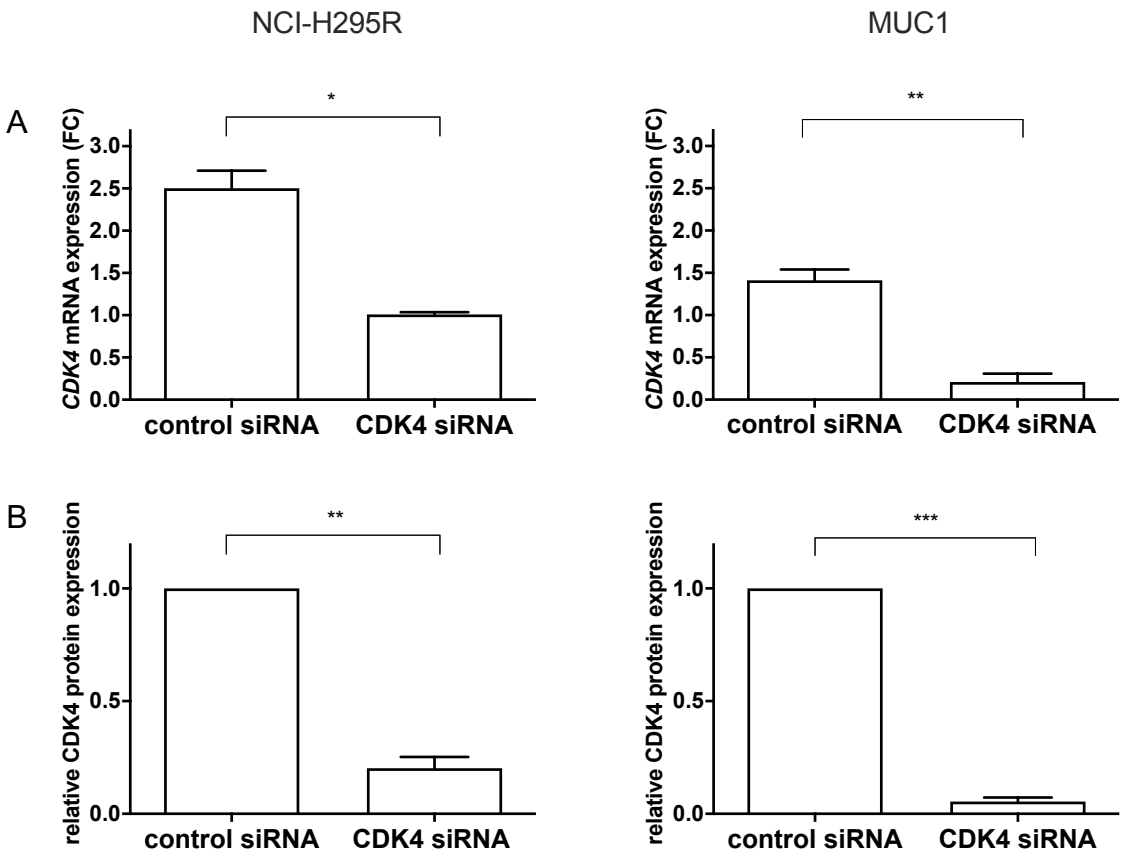


Figure 3.11: CDK4 mRNA and protein expression after CDK4 siRNA treatment in two ACC cell lines.

A) Comparison of relative CDK4 mRNA FC by RT-qPCR 72 h after CDK4 knockdown with CDK4 siRNA and treatment with control siRNA in the NCI-H295R (left) and MUC1 (right) cell line. β-actin was used as the housekeeping gene. Experiments were conducted in triplicates.

B) Comparison of relative CDK4 protein expression by quantitative WB analysis 72 h after CDK4 knockdown. α-tubulin was used as internal standard. Each corresponding control was defined as 1.0, each bar of the histograms represents the relative ratio of CDK4 to α-tubulin signal. Experiments were conducted in triplicates.

Statistical analysis by unpaired t-test with Welch‘s correction. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$.

This figure was modified according to Liang et al., 2020.

Changes in cell viability through CDK4 knockdown were then investigated by WST1 assay. A clear reduction of cell viability was observed in CDK4 siRNA transfected NCI-H295R cells in comparison with the corresponding control (88.6 ± 9.3 *vs* 100.0 ± 8.6, $p < 0.0001$) while MUC1 cells showed no significant effect after transfection (97.0 ± 7.6 *vs* 100.0 ± 6.9, $p = 0.175$) (figure 3.12).

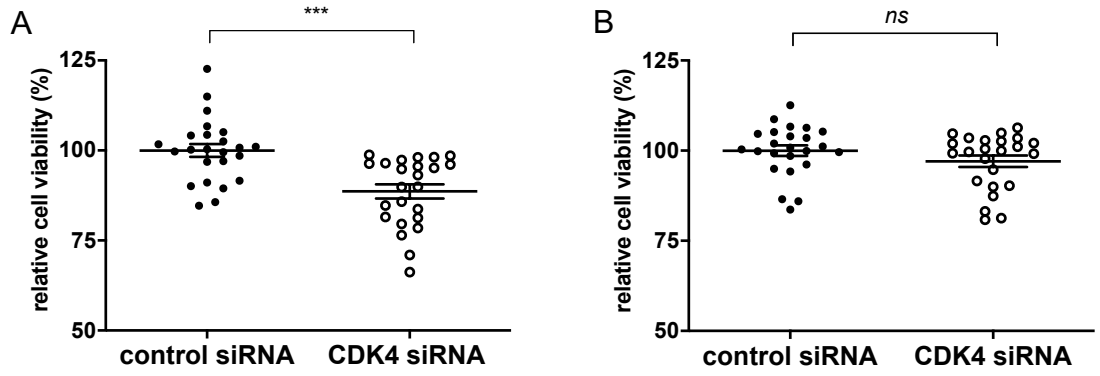


Figure 3.12: Cell viability after CDK4 siRNA treatment in two ACC cell lines.
A) Cell viability measured by WST1 test 72 h after CDK4 knockdown in the NCI-H295R cell line. The mean of the absorbance measured for cells transfected with control siRNA was defined as 100% for each experiment. The ratio of measured absorbance to the mean absorbance forms the final data. Three independent experiments were conducted using octuplet samples.
B) Cell viability after CDK4 knockdown in the MUC1 cell line conducted as described above. Three independent experiments were conducted using octuplet samples.
Statistical analysis by unpaired t-test with Welch's correction. ns = p not significant, ***$p < 0.001$.
This figure was modified after Liang et al., 2020.

3.6.3. Treatment with CDK4/6 inhibitor palbociclib

Both cell lines were then treated by the CDK4/6 inhibitor palbociclib in increasing concentrations to examine the *in vitro* drug effect on ACC cells. A time- and dose-dependent decrease of cell viability was observed. Palbociclib treatment in NCI-H295R cells achieved a significant reduction of cell viability using concentrations of as low as 2 µM after longer time intervals (96 and 192 h) while administration of higher drug concentrations as 16 µM was already able to cause notable reduction of cell viability at 48 h compared to vehicle treated cells. This effect was further enhanced at later time points (82.3 ± 5.2 *vs* 50.5 ± 19.2 *vs* 48.9 ± 4.3 *vs* 19.1 ± 4.6 at 48, 96, 144, 196 h, respectively, using 16 µM palbociclib, all $p < 0.001$) (figure 3.13 A). Similarly, in MUC1 cells, a decrease of cell viability was set off at a minimum of 2 µM after 96 h and viability was reduced at all time points using drug concentrations of 8 and 16 µM (81.7 ± 3.0 *vs* 74.1 ± 8.7 *vs* 63.8 ± 17.7 *vs* 53.1 ± 10.9 at 48, 96, 144, 196 h, respectively, using 16 µM palbociclib, all $p < 0.001$) (figure 3.13 A). Comparison of the general impact of palbociclib treatment on cell viability in the two cell lines showed a stronger reduction in NCI-H295R than in MUC1 cells. This effect became more distinct at longer treatment times and higher drug concentrations (figure 3.13 B).

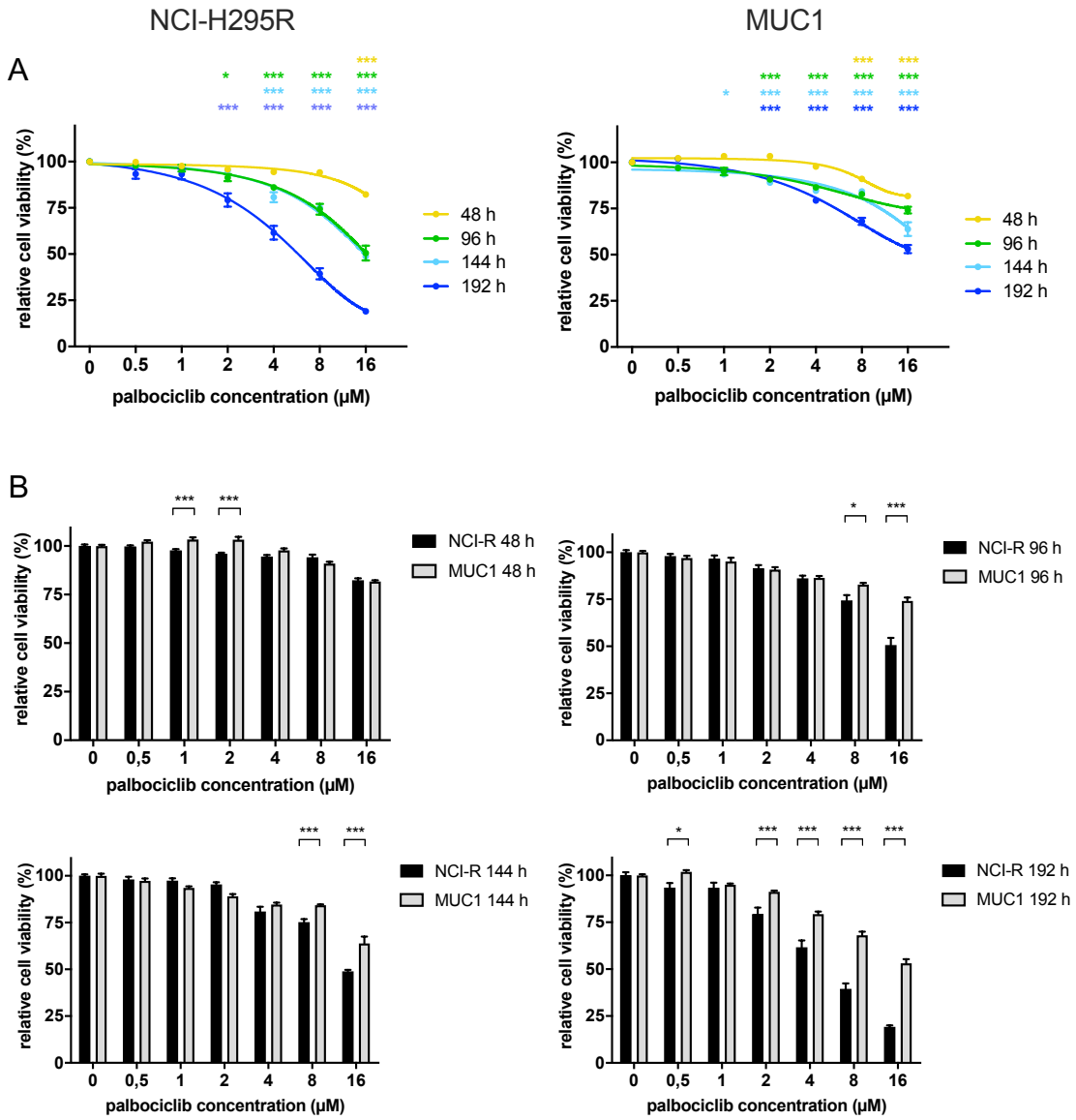


Figure 3.13: Treatment with CDK4/6 inhibitor palbociclib in two ACC cell lines.

A) Interpolation of relative cell viability measured by WST1 test in 48-hour intervals after treatment with increasing concentrations of palbociclib in the NCI-H295R (left) and MUC1 (right) cell line. The mean of the absorbance measured for cells treated with DMSO was defined as 100% for each experiment. The ratio of measured absorbance to the mean absorbance forms the final data. For both cell lines three independent experiments were conducted using octuplet samples.

B) Comparison of cell viability in NCI-H295R and MUC1 cell lines after palbociclib treatment at 48, 96, 144 and 192 h. Cells were treated as outlined above. For both cell lines three independent experiments were conducted using octuplet samples.

Statistical analysis by two-way ANOVA. *$p < 0.05$, ***$p < 0.001$ for comparison with the control sample.

This figure was modified after Liang et al., 2020.

3.6.4. Treatment with palbociclib in combination with IGF1R/IR inhibitor linsitinib

The IGF1 pathway is frequently overactivated in ACC. Application of linsitinib, a dual inhibitor of IGF1R and IR, successfully lead to a decrease in cell viability in several ACC cell experiments (Peixoto Lira et al., 2016, Barlaskar et al., 2009) but failed in a phase III clinical trial for ACC patients (Fassnacht et al., 2015). As it is described for IGF signaling to lead to a CDK4/6 activation (Tang et al., 2017), a combination of the IGF1R/IR inhibitor linsitinib and CDK4/6 inhibitor palbociclib was tested in the two ACC cell lines. Single treatment with linsitinib decreased cell viability time- and dose-dependently in both ACC cell lines (figure 3.14 A and B) as previously reported. The effect of the combination of 0.25 µM linsitinib and 4 µM palbociclib was tested at two time points, 48 h and 96 h.

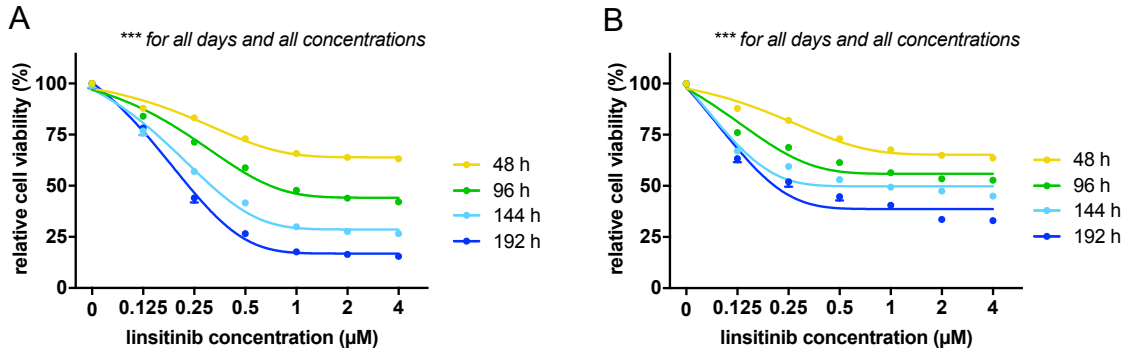


Figure 3.14: Treatment with IGF1R/IR inhibitor linsitinib in two ACC cell lines.
A) Interpolation of relative cell viability measured by WST1 test in 48-hour intervals after treatment with increasing concentrations of linsitinib in the NCI-H29R cell line. The mean of the absorbance measured for cells treated with DMSO was defined as 100% for each experiment. The ratio of measured absorbance to the mean absorbance forms the final data. For both cell lines three independent experiments were conducted using octuplet samples.
B) Interpolation of relative cell viability after treatment with linsitinib in the MUC1 cell line conducted as described above. For both cell lines three independent experiments were conducted using octuplet samples.
Statistical analysis by two-way ANOVA. ***$p < 0.001$ for comparison with the control sample.
This figure was modified after Liang et al., 2020.

At 48 h, a significant decrease of cell viability in combination treated NCI-H295R cells was observed compared to both single drug treatments (40.5 ± 9.7 *vs* 54.7 ± 6.5 and 66.3 ± 15.6, both $p < 0.001$ for combination *vs* linsitinib and palbociclib treatment, respectively) while at 96 h a significant effect sustained only in comparison to palbociclib

treatment (51.4 ± 5.8 *vs* 50.5 ± 4.2 and 66.0 ± 12.1 for combination *vs* linsitinib and palbociclib treatment, respectively, ns and $p < 0.001$) (figure 3.15 A). In MUC1 cells, viability of combination treated cells was significantly lower at 48 h in respect to treatments with linsitinib or palbociclib only (74.9 ± 3.6 *vs* 78.5 ± 3.1 and 97.7 ± 4.8, $p < 0.05$ and $p < 0.001$ for combination *vs* linsitinib and palbociclib treatment, respectively). At 96 h, the significance on the reduction in cell viability through the combination treatment was further enhanced compared to linsitinib treatment (59.8 ± 3.8 *vs* 68.0 ± 3.8, $p < 0.001$) while remaining unchanged in compared to palbociclib treatment (84.3 ± 2.3, $p < 0.001$) (figure 3.15 B).

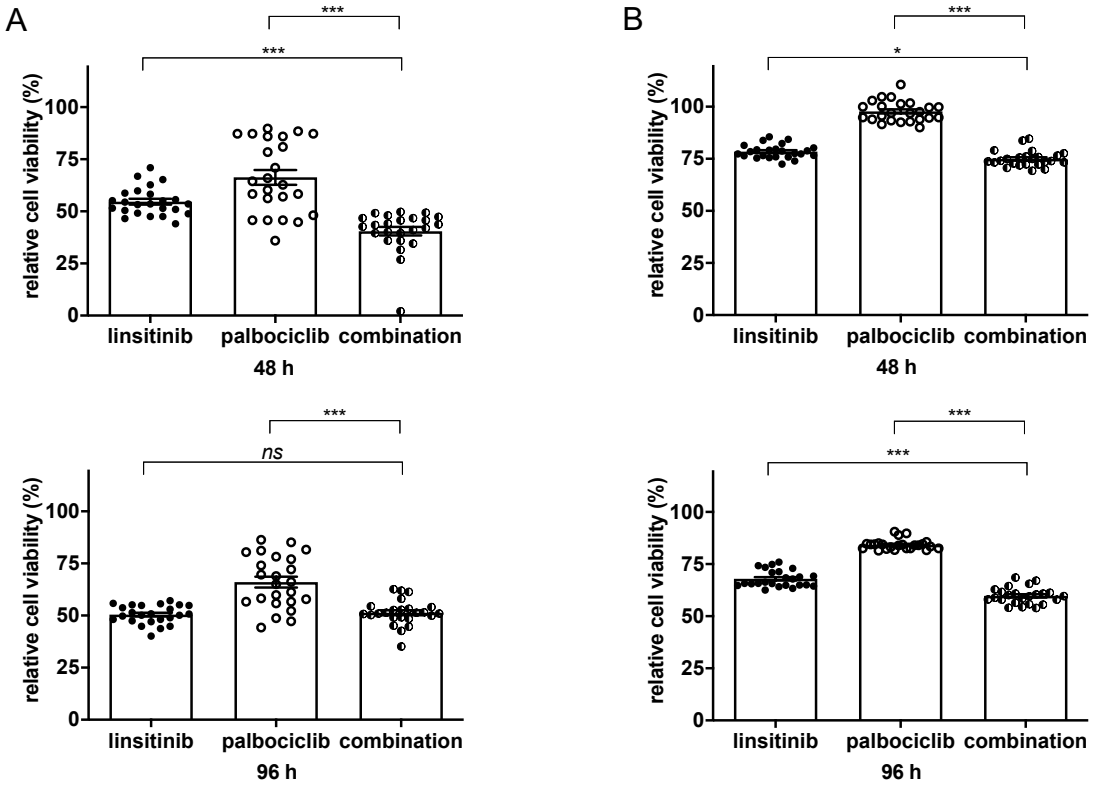


Figure 3.15: Effect of treatment with a combination of CDK4/6 inhibitor palbociclib and IGFR/IR inhibitor linsitinib on cell viability in two ACC cell lines.

A) Relative cell viability obtained as described in figure 3.13 under administration of 0.25 µM linsitinib and 4 µM palbociclib in NCI-H295R cells after 48 h and 96 h.

B) Relative cell viability obtained as described in figure 3.13 under administration of 0.25 µM linsitinib and 4 µM palbociclib in MUC1 cells after 48 h and 96 h.

Statistical analysis by one-way ANOVA. ns = p not significant, *p < 0.05, ***p < 0.001.

3.6.5. Treatment effect on CDK4 pathway in ACC cells

To gain insight into the effect of the treatments on the CDK4 pathway protein expression of CDK4, $p16^{INK4A}$ and p130/RBL2 was analyzed in the two ACC cell lines before and

after single treatment with palbociclib and linsitinib as well as the combination of both drugs (figure 3.16 A). While expression of CDK4 and $p16^{INK4A}$ remained unchanged by the different treatments, a reduction in p130/RBL2 expression was measured after palbociclib and combined treatment in both cell lines NCI-H295R and MUC1 (figure 3.16 B). No difference in p130/RBL2 expression was detected after linsitinib treatment.

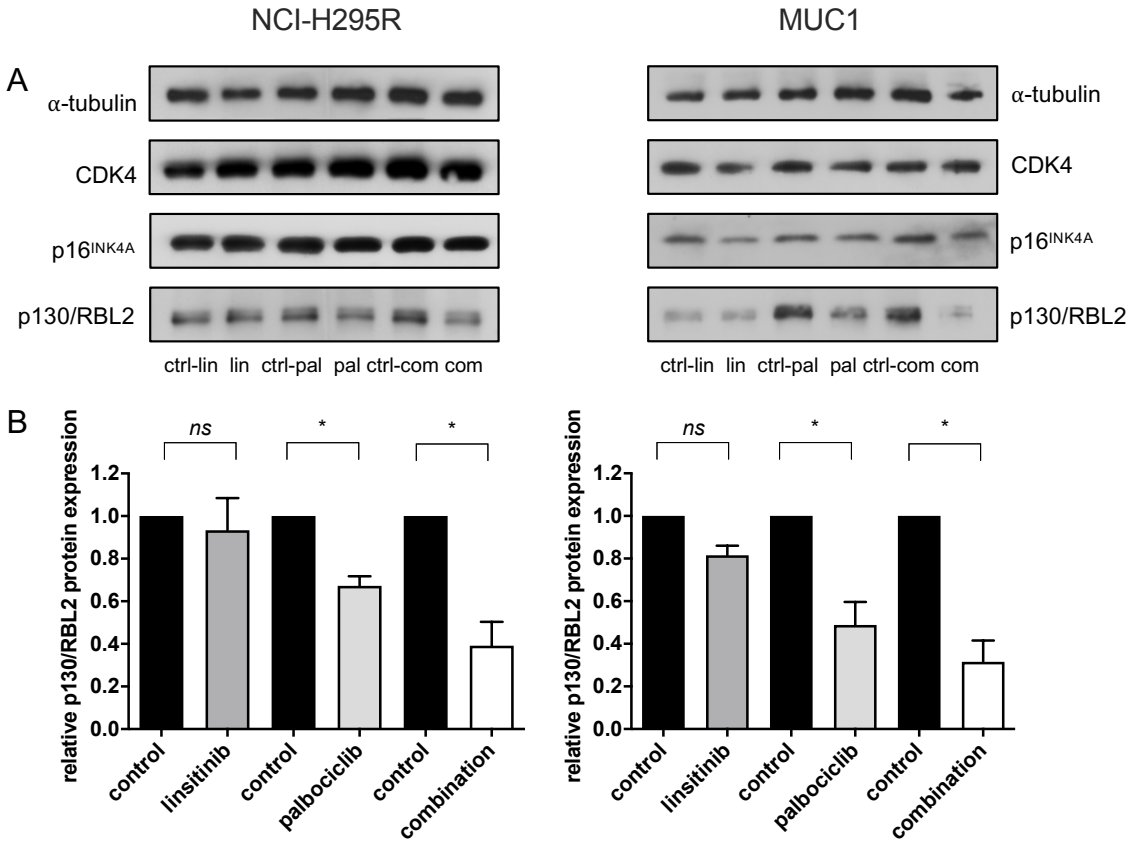


Figure 3.16: Effect of treatment with a combination of CDK4/6 inhibitor palbociclib and IGFR/IR inhibitor linsitinib on protein expression of CDK4-related factors in two ACC cell lines.

A) WB analyses show little effect after drug treatment for 96 h on protein expression of CDK4 and $p16^{INK4A}$ but decreasing levels of p130/RBL2 after palbociclib treatment as well as after treatment with the combination of palbociclib and linsitinib in both cell lines. The blot is representative for three independent experiments. ctrl-lin = correspondent control to linsitinib treatment, lin = linsitinib, ctrl-pal = correspondent control to palbociclib treatment, pal = palbociclib treatment, ctrl-com = correspondent control to combination treatment, com = combination of palbociclib and linsitinib.

B) Quantitative WB analyses of p130/RBL2 in linsitinib, palbociclib and combination treated cells. α-tubulin was used as the internal standard. Each corresponding control was defined as 1.0, each bar of the histograms represents the relative ratio of p130/RBL2 to α-tubulin signal after normalization to the control. Each experiment was conducted in triplicates. ns = p not significant, *$p < 0.05$. Statistical analysis by unpaired t-test with Welch's correction.

This figure was modified after Liang et al., 2020.

4. Discussion

To date, treatment for patients with advanced ACC remains a therapeutic challenge with only a minority of them gaining major profit from conventional systemic cytotoxic therapy despite severe adverse effects. No effective targeted therapy options have been established regardless of considerable efforts and some promising pre-clinical results (Altieri et al., 2020).

Aiming to identify new potential druggable genes and targeted treatment strategies feasible for possible administration in the clinical setting, targeted gene expression profiling of known cancer drug targets was carried out on 40 FFPE tumor samples.

4.1. Feasibility of targeted RNA sequencing from FFPE samples in ACC

RNA was extracted from FFPE specimen which are easily available in the clinical setting as opposed to frozen tissue, for which mRNA extraction is well established. RNA isolation was performed 104 FFPE specimen with 40 of 104 samples being adequate for further RNA analyses as verified by the quality check of obtained RNA. Gene expression of 84 potential drug targets was then investigated through targeted RNA screening identifying promising potentially targetable gene families such as AURK, CDK and PLK and candidate drug target genes such as CDK1, CDK4, PLK1 and TOP2A.

Viability of the extraction method using FFPE tissue was tested by mRNA expression of five random samples from both FFPE specimen and frozen tissue in the RT2 Profiler. Testing these corresponding samples produced concordant results regarding gene expression in the RT2 Profiler, thus verifying the feasibility of this method. However, good quality RNA isolation from FFPE material was obtained in only 38% of cases rendering the majority of FFPE samples unfit for further targeted RNA analyses. Low quality or low quantity as a result of RNA degradation due to formalin fixation is a common problem despite several pre-analytical methods being investigated for improvement of RNA quality (Marczyk et al., 2019, Adiconis et al., 2013). Among others, storage time as well as exposure of FFPE samples to air and light are reported to influence RNA quality and RNA isolation within one year after fixation and embedding is recommended (von Ahlfen et al., 2007). Therefore, it would be conceivable to achieve better results by early RNA isolation from ACC FFPE specimen for further RNA profiling if applied to clinical practice.

4.2. Identification of promising drug targets

Through targeted gene expression profiling promising actionable gene families such as the CDK, PLK and AURK family and six potential candidate genes with overexpression in at least 50% of ACC cases were identified.

4.2.1. CDK4

CDK4 was chosen for further investigation as best potential drug target according to our pre-selection criteria. Indeed, *CDK4* overexpression was observed in 62% of ACC samples and *CDK4* mRNA expression significantly correlated with the presence of CN gains at DNA level (Lippert et al., 2018). Moreover, CDK4/6 inhibitors such as palbociclib are already FDA-approved for specific types of breast cancer and under ongoing clinical trials for other solid tumors, thereby meeting a crucial criterion for further examination. Supporting the choice of this drug target, promising results were obtained by CDK4/6 inhibitors previously tested in ACC cell lines (Fiorentini et al., 2018).

4.2.1.1. Association between CDK4 protein expression and clinical outcome

CDK4 protein expression analyzed by IHC was significantly associated with *CDK4* mRNA expression in the entire cohort of 104 patients. Moreover, a relationship between longer PFS and high CDK4 protein expression was observed, but not with CDK4 gene expression.

Similarly, in a variety of tissues, CDK4 protein expression was reported to be mainly consistent with *CDK4* mRNA expression (www.proteinatlas.org). Regarding survival rates, a similar relationship was reported in high-risk endometrial cancer (Ikeda et al., 2015) suggesting a more favorable prognosis in patients with high CDK4/6 specific activity. However, in general, CDK4 expression is reported to be linked to poor clinical outcome in the majority of solid tumors (Ismail et al., 2011). Additionally, we observed a correlation between high CDK4 immunoreactivity and longer TTP in platinum compound treated patients (n = 53) which could contribute to the effect of the more favorable PFS rate. CDK4 was previously reported to be one of the targets of cisplatin with high CDK4 expression and could therefore potentially be involved in better response to therapy with platinum compounds (He et al., 2011).

Therefore, a larger cohort is undoubtedly necessary for a sound assessment on the prognostic role of CDK4 in ACC patients.

4.2.1.2. Rationale for use of CDK4/6 inhibitors in selected ACC patients

Even if a relationship between high CDK4 expression and favorable outcome was observed, a strong rationale remains for the use of CDK4/6 inhibitors in ACC patients with high CDK4 expression regardless. Though disease relapse might then occur later than patients with low CDK4 expression, about 50% of patients experience recurrence within the first two years after primary surgery with a fair share needing effective palliative pharmacological therapy.

CDK4/6 inhibitors (i.e. abemaciclib, palbociclib, ribociclib) were recently approved for HR-positive/HER2-negative advanced breast cancer in combination with hormone therapy (Deng et al., 2018, Ramos-Esquivel et al., 2018). At present, CDK4/6 inhibitors are tested in phase I and II clinical trials on several other solid tumors (Patnaik et al., 2016, Hamilton and Infante, 2016) as well as in preselected patients with tumors presenting amplification or overexpression of CDK4 (Dickson et al., 2013) (NCT03242382).

4.2.1.3. Targeting CDK4 in cell lines

To test the effects of CDK4 inhibition in ACC cells, *in vitro* functional studies were performed using the CDK4/6 inhibitor palbociclib in two available ACC cell lines NCI-H295R and MUC1 (Hantel et al., 2016) with different molecular pattern. Palbociclib led to an impairment of cell viability which was more evident in NCI-H295R cells than in MUC1 cells.

Our findings about the effects of palbociclib on NCI-H295R cells are in line with two recent studies in ACC cell lines. With one study testing palbociclib under consideration of *CDK4* and *CDK6* mRNA overexpression in NCI-H295R and SW13 cells (Fiorentini et al., 2018) and a second study analyzing the effect of palbociclib in conjunction with CDK6 expression (Hadjadj et al., 2017), the present experiments confirmed a concentration-dependent decrease of cell viability through palbociclib in NCI-H295R cells. Additionally, we could show a similar effect in the lately established ACC cell line

MUC1 for the first time. Moreover, a time-dependent effect was observed in both cell lines.

Interestingly, palbociclib led to significantly stronger impairment of cell viability in NCI-H295R cells than in MUC1 cells. Of note, NCI-H295R cells displayed higher CDK4 expression at both mRNA and protein level as well as a significant reduction of cell viability after CDK4 knockdown. As this stands in contrast to MUC1 cells which showed no significant impairment of cell viability after CDK4 knockdown it may be hypothesized that the difference in viability might be explained by level of CDK4 expression. Therefore, the observation regarding the difference in sensitivity to palbociclib as CDK4/6 inhibitor might be at least partly dependent on CDK4 expression.

It should be noted though that relatively high drug concentrations (i.e. 1-5 µM) were administered to ACC cells in current and former studies on palbociclib. As these would be difficult to maintain in patients, further investigations in animal models are needed to confirm feasibility of palbociclib treatment for ACC patients.

4.2.1.4. p130/RBL2 as substitute for RB

No RB protein expression was observed in neither of the two tested ACC cell lines. Despite the lack of RB expression as effector protein of CDK4, a decrease in cell viability following palbociclib treatment was observed in both cell lines and p130/RBL2 was proposed as a potential substitute for RB protein.

Consistent with these findings, RB protein was found to be absent in NCI-H295R cells in a previous study (Fiorentini et al., 2018) with previous reports on ACC tumor tissues describing deletions of the *RB1* gene and a lack of RB in 7% of cases (Assie et al., 2014). With the effect of CDK4/6 inhibitors like palbociclib typically closely linked to RB phosphorylation, this may arise the question of the underlying effect mechanism of palbociclib in ACC cells.

However, it is known that the CDK4 downstream pathway also includes other members of the related RB-like family serving as substrates for CDK4/6 as well (see figure 2.2) (Malumbres and Barbacid, 2005). For instance, p130/RBL2, a member of the RB-like family involved in cell cycle arrest and senescence, was previously found to be expressed in NCI-H295R cells (Fiorentino et al., 2009, Fiorentini et al., 2018, Hadjadj et al., 2017, Rivadeneira et al., 2010) and chosen as a potential effector protein of the mechanism of

action of palbociclib. Indeed, clear p130/RBL2 expression was detected in both NCI-H295R and MUC1 cells. Similar to the reduction of pRB in the palbociclib treated ACC cell line SW13 due to decrease of phosphorylated protein (Hadjadj et al., 2017), a reduction of p130/RBL2 after treatment was observed in NCI-H295R and MUC1 cells indicating that CDK4 inhibition by palbociclib is potentially responsible for activation of p130/RBL2. It seems therefore reasonable that ACC cells are sensitive to palbociclib through p130/RBL2 expression, similar to RB negative palbociclib sensitive hepatoma cells (Rivadeneira et al., 2010), and ACC patients could benefit from compounds targeting CDK4/6 in ACC regardless of *RB1* expression.

4.2.1.5. Combination of CDK4/6 and IGF1R/IR inhibitors

Linsitinib and palbociclib were used for simultaneous inhibition of CDK4/6 and IGF1R/IR achieving a stronger reduction of cell viability than accomplished by the single drug in both NCI-H295R and MUC1 cells.

Despite IGF1R/IR inhibitors being reported as promising targeted therapy for ACC in *in vitro* experiments (Barlaskar et al, 2009), these encouraging findings could not be confirmed in a clinical setting (Fassnacht 2015). IGF signaling activation is reported to lead to upregulation of cyclin D1 by the PI3K (phosphatidylinositol-3-kinase)/AKT (protein kinase B) pathway (Tang et al., 2017). This resistance mechanism provides a potential reason for the inadequate response in ACC patients treated with the IGFR/IR inhibitor linsitinib. In several tumor cell lines a drug combination targeting both the IGF1R and CDK4 pathway are observed to be more efficient than using the singe drug while showing a correlation with reduced mTORC1 (mammalian target of rapamycin complex 1) activity (Miller et al., 2013, Guenther et al., 2019, Heilmann et al., 2014, Sherr, 2016), as was observed in the present study. In addition, IGF1R activation was described as an escape mechanism to CDK4/6 inhibitors in Ewing Sarcoma and dual targeting of CDK4/6 and IGF1R inhibitors could constitute a candidate synergistic combination (Guenther et al., 2019). Presently, a phase Ib clinical trial with an IGF1/IGF2 antibody and CDK4/6 inhibitor is ongoing in different solid tumors (NCT03099174).

To date, mitotane or EDP-M is recommended as first-line treatment of advanced ACC (Fassnacht et al., 2012). Mitotane is generally known for its toxicity and its narrow therapeutic range with adverse reactions often limiting the aspired dosage. Common

adverse effects are gastrointestinal disorders and neurological effects (Fassnacht et al., 2018). As described before (see section 1.2.6.3), EDP-M currently constitutes the most validated option for aggressive advanced ACC, however, response rates even under this regimen remain low. Adverse events mostly include hematological, gastrointestinal and neurological effects (Fassnacht et al., 2012, Fassnacht et al., 2018). Both palbociclib and linsitinib have displayed overall good tolerance in patients with the most common adverse events being hematologic toxicity under palbociclib (Cristofanilli et al., 2016), while patients receiving linsitinib show fatigue, nausea and hyperglycemia under linsitinib (Fassnacht et al., 2015). Therefore, even a combination of palbociclib and linsitinib remains theoretically competitive compared to EDP-M.

4.2.2. IGF family

IGF2 overexpression was detected in 95% of cases in the present series while *IGF1R* expression was moderate and downregulated in 45% of cases. Examination of the ACC cell lines NCI-H295R and MUC1 demonstrated overexpression of both *IGF2* and *IGF1R*. IGF2 binds to IGF1R and IR to activate the PI3K/AKT/mTOR pathway leading to cell proliferation and development. In line with present findings, IGF signaling is frequently reported to be activated in about 80-90% and *IGF2* gene itself is well known to be overexpressed in ACC (Altieri et al., 2019, Ribeiro and Latronico, 2012, Giordano et al., 2009). Though the specific dual IGFR/IR inhibitor linsitinib (OSI-906) showed strong effects *in vitro* using ACC cell lines as well as *in vivo* using tumor xenografts in mice (Barlaskar et al., 2009), the drug failed to achieve a significant efficacy when investigated in clinical trials. However, it should be noted that disease control was achieved from 23 to > 45 months in 4 patients (4.4%) with 3 of the patients showing partial response to linsitinib treatment. No predictive factor in line of demographic, clinical, histopathological or pharmacological criteria has been identified for response to IGF1R inhibition. With unknown genomic background of the patients before treatment, ongoing molecular characterization of tumor samples of treatment responders and non-responders aim to disclose a potential predictive signature for sensitivity to linsitinib to improve patient selection (Fassnacht et al., 2015). Moreover, ACC tumorigenesis could be driven by multiple pathway alterations. *In vivo* studies suggest that tumorigenesis could not be achieved by IGF2 overexpression alone but through additional alterations, such as

activation of β-catenin signaling (Heaton et al., 2012). Therefore, it seems possible that combination targeted therapies are required (Costa et al., 2016). A phase I study investigated the combination of IGF1R inhibitor cixutumumab with mTOR inhibitor temsirolismus achieving stable disease in 42% of patients (Naing et al., 2013).

In our ACC cohort, *IGF1R* gene, the main target of linsitinib, was upregulated in 5% and down-regulated in 45% of cases, similarly to what was described in a previous series (Peixoto Lira et al., 2016). On the contrary, an *IGF1R* overexpression has been observed in NCI-H295R cells in both present and previous studies (Peixoto Lira et al., 2016, Barlaskar et al., 2009). The modest or absent expression of IGF1R in ACC tissue samples might therefore constitute a potential reason for the mostly disappointing outcome in ACC. Pre-screening for *IGF1R* overexpression might help to pre-select patients that may benefit from therapeutic option with linsitinib.

4.2.3. CDK1

CDK1 gene expression was upregulated in 80% of cases in the present series. A significant correlation with protein expression was observed reflecting reports on CDK1 protein expression in other tissues (www.proteinatlas.org). While *CDK1* mRNA expression was significantly related to OS and PFS (n = 40), the significance did not remain for CDK1 protein expression (n = 104).

CDK1 as a cyclin-dependent kinase is essential for G1/S and G2/M phase transitions and is therefore involved in cell cycle control and cell proliferation (Whittaker et al., 2017). Accordingly to the present series, *CDK1* mRNA overexpression was described for different cohorts of ACC patients (Nilubol et al., 2018, Glover et al., 2015) and was found to be associated with poor survival (n = 92 and n = 34 for the The Cancer Genome Atlas database and the European Bioinformatics Institute database, respectively (Nilubol et al., 2018)).

Suppression of CDK1 in ACC cells was examined by multi-targeting microRNA-7 (Glover et al., 2015) and a combination of a proteasome inhibitor with broad-spectrum CDK inhibitor flavopiridol (Nilubol et al., 2018) achieving an impact on cell proliferation in both cases. Specific CDK1 inhibitors have not yet entered clinical trials while flavopiridol and small molecule inhibitors of CDK1/2 and, to lesser extent, other CDKs

such as dinaciclib and roscovitine are under investigation in early clinical trials (Carvajal et al., 2009, Lin et al., 2009, Mita et al., 2017, Cicenas et al., 2015).

4.2.4. PLK1

In the present cohort, *PLK1* gene expression was upregulated in 52%. A significant relationship was observed between PLK1 mRNA and protein expression similarly as described in rectal cancer (Rodel et al., 2010). No significant relationship was found between PFS or OS data and *PLK1* gene (n = 40) or PLK1 protein expression (n = 104).

PLK1 is a serine/threonine kinase with an important role in the regulation of cell cycle controlling CDK1/cyclin B activity and transition into mitosis as well as acting as a negative regulator of p53 among other things (Liu, 2015). In the cohort published by (Bussey et al., 2016) (n = 44), an upregulation of *PLK1* was reported in 29% of ACC cases, while a re-analysis of the cohort published by Giordano et al., 2009 (n = 33) demonstrated an upregulation in 67% of cases with the percentage of *PLK1* upregulation in the present cohort being close to the middle value. As opposed to our data, however, both cohorts combined showed an association between high *PLK1* expression and poor prognosis (Bussey et al., 2016). Investigation in a larger cohort may help to clarify the discrepancy.

Both inhibition of PLK1 through PLK1 siRNA and small molecule inhibitor BI 2536 targeting PLK1 achieved decrease of viability in two ACC cell lines, NCI-H295R and SW-13, as well as in xenograft mice models (Bussey et al., 2016). Of note, SW13 showed a better response than NCI-H295R cells, presumably due to p53 status as SW13 cells harbor a mutant p53 protein while NCI-H295R cells present wild type p53.

Several PLK1 inhibitors are currently under clinical investigation. While BI 2536, an early PLK1 inhibitor, achieved mixed results in phase II clinical trials (Mross et al., 2012, Muller-Tidow et al., 2013), more recent PLK1 inhibitors like volasertib and rigosertib are under investigation in phase II and III clinical trials in solid and non-solid tumors (e.g. NCT01721876, NCT01360853). Moreover, a phase I/II study of PLK1 inhibitor TKM-080301 is ongoing in neuroendocrine tumors and adrenocortical carcinoma patients (NCT01262235) (Bussey et al., 2016). Altogether, these data demonstrate that PLK1 inhibitors might represent a promising targeted treatment for selected ACC patients.

4.2.5. TOP2A

In the present ACC cohort, a strong upregulation of *TOP2A* gene expression could be confirmed, as previously reported in previous series (Giordano et al., 2003, Jain et al., 2013). No significant relationship was found between *TOP2A* gene expression and protein expression or between TOP2A expression and survival rates.

Topoisomerases are responsible for DNA strand breaks to reduce supercoiling and are therefore essential to DNA transcription and replication. TOP2A as a topoisomerase 2 isoform introduces double-strand breaks in the S, G2 and M phase of the cell cycle. While a significant correlation between TOP2A gene and protein expression was reported by Roca et al., 2017 (n = 39), this finding was not confirmed in our cohort. This discrepancy could be further investigated in larger cohorts. Moreover, TOP2A protein expression was also previously associated with poorer overall and disease free survival (n = 61) (Ip et al., 2015), which was not observed our cohort (n = 104 by IHC).

Indeed, general TOP2-poisons like etoposide and doxorubicin are already deployed in the EDP-M regimen routinely used for ACC patients with advanced disease showing an advantage in PFS and response rates in comparison to streptozotocin combined with mitotane though general response rates remain low (Fassnacht et al., 2012). Considering that extra-tumoral toxicities are often dose-limiting in cytotoxic therapies, novel catalytic inhibitors for TOP2, like aclarubicin, currently under investigation in phase I and II clinical trials in leukemia patients have been reported to be overall well tolerated (Song et al., 2011) and to have potent anticancer effect in ACC cell lines (Jain et al., 2013). Moreover, *TOP2A* gene expression was suggested as a predictive marker for response to EDP-M therapy itself (Roca et al., 2017). While statistical analysis regarding this relationship failed to confirm this finding in the present treated subcohort, it should be noted that both studies are limited by insufficient number of patients (n = 53 and n = 26 for the present series and the series by (Roca et al., 2017), respectively) and a larger cohort is needed to assure a more reliable assessment.

4.3. Application to clinical practice

The aim of this study was the identification of potential new drug targets and treatment strategies with methods easily transferable to the clinical routine. Therefore, targeted

RNA profiling was performed on FFPE material. As good quality RNA isolation from FFPE material was achieved in 38% of cases, it is important to underline that routine assessment of protein expression of the drug target candidates CDK1, CDK4 and PLK1 in ACC samples is feasible through IHC. In particular, it appears possible to preselect ACC patients that could benefit from treatment with CDK4/6 inhibitors like palbociclib based on presence of CDK4 CN gains on DNA level and/or CDK4 overexpression at mRNA or protein level in the tumor. Considering that the *CDK4* gene was affected by CN gains in 40% and overexpressed in > 60% of cases of ACC samples in the present cohort, screening of candidate patients for *CDK4* gain amplification or overexpression by RT-qPCR or by IHC, according to local availabilities and facilities, might be proposed. Moreover, with a lack of RB in 7% of cases reported (Assie et al., 2014) present and previous findings suggest that CDK4/6 inhibitors could function as ACC treatment irrespective of RB expression as sensitivity of ACC cells to palbociclib could also be due to p130/RBL2 expression.

Taking into account that 37,5% of cases presented high *CDK4* and normal or high *IGF1R* mRNA levels, combined treatment with CDK4/6 inhibitor palbociclib and IGF1R/IR inhibitor linsitinib might be an interesting combination partner in this subgroup of patients.

4.4. Strengths and limitations

This study demonstrates several strong points besides certain limitations.

One of the limitations of this study is the limited number of patients in comparison with other tumor entities, in particular for RNA analyses. Considering the low incidence of ACC, however, the RNA cohort consisting of 40 patients fits within the scale of past mid-scale molecular screening studies of ACC (De Martino et al., 2013, Ross et al., 2014). Aiming for extension of the range of involved ACC and additional support of the results from present mRNA analyses, CDK4 gene expression available from a previously published transcriptome data set was investigated obtaining similar findings. Moreover, results obtained in the RNA cohort were further investigated on protein level in a cohort consisting of 104 patients (Lippert et al., 2018, Roca et al., 2017, Jain et al., 2013, Laufs et al., 2018). Nonetheless, validation of important findings is needed. To this end, a

validation study in an independent prospective cohort of ACC patients could provide further insight.

Another limitation concerns the retrospective study design though potential biases were minimized by defined inclusion criteria of patients with detailed clinical data and adequate quantity of tumor material. Again, investigation of a prospective cohort is needed for validation.

In addition, targeted mRNA profiling was used in this thesis. While covering a broad range of potential cancer drug targets, it certainly lacks information in comparison to whole genome sequencing. With the emphasis of this study being on the applicability to clinical routine and after diligent choice of a panel of drug targets of interest, the decision was made in favor of targeted analyses.

Immunohistochemical stained slides were evaluated by intensity of staining and percentage of positive cells with inter- and intra-observer variability. However, two independent operators blinded to the results and clinical information were able to achieve mostly consistent results (Pearson correlation coefficient 0.53-0.79).

Additionally, only two ACC cell lines were used for *in vitro* functional studies which might appear expandable in comparison with other tumor cell lines. With a general lack of established ACC cell lines, NCI-H295R and MUC1 cells were chosen as two molecularly different ACC cell lines with different CDK4 expression to be investigated. Of course, future investigations in viable cells or animal models can offer further affirmation of present findings.

Aiming at a future personalized management of ACC patients with pre-screening of patients according to the molecular pattern, this study represents an innovative approach for ACC that has never been clearly proposed until now for clinical practice. In particular, it might serve as a basis for a clinical trial on CDK4/6 inhibitors alone or in combination with IGF1R/IR inhibitors in a pre-selected group of patients with advanced ACC.

5. Summary

Adrenocortical carcinomas (ACC) are aggressive tumors associated with a heterogeneous but generally poor prognosis and limited treatment options for advanced stages. Despite promising molecular insights and improved understanding of ACC biology, efficient targeted therapies have not been identified yet. Thus, this study aims to identify potential new drug targets for a future personalized therapeutic approach.

RNA was isolated from 104 formalin-fixed paraffin-embedded tumor samples from ACC patients, 40 of those 104 cases proved to be suitable for further mRNA analyses according to the quality check of the extracted RNA. Gene expression of 84 known cancer drug targets was evaluated by quantitative real-time PCR using 5 normal adrenal glands as reference. Protein expression was investigated for selected candidate drug targets by immunohistochemistry in 104 ACC samples, 11 adenomas and 6 normal adrenal glands. Efficacy of an available inhibitor of the most promising candidate was tested by functional *in vitro* experiments in two ACC cell lines (NCI-H295R and MUC1) alone or in combination with another drug.

Most frequently overexpressed genes were TOP2A, IGF2, CDK1, CDK4, PLK4 and PLK1. Nuclear immunostaining of CDK1, CDK4 and PLK1 significantly correlated with the respective mRNA expression. CDK4 was chosen as the most promising candidate for functional validation as it is actionable by FDA-approved CDK4/6 inhibitors. ACC samples with copy number gains at CDK4 locus presented significantly higher CDK4 expression levels. The CDK4/6 inhibitor palbociclib showed a time- and concentration-dependent reduction of cell viability *in vitro*, which was more pronounced in NCI-H295R than in MUC1 cells. This was in line with higher CDK4 expression at western blot analysis in NCI-H295R cells. Furthermore, palbociclib was applied in combination with dual IGFR/IR inhibitor linsitinib showing a synergistic effect on reducing cell viability.

In conclusion, this proof-of-principle study confirmed RNA profiling to be useful to discover potential drug targets. Detected drug targets are suitable to be investigated by immunohistochemistry in the clinical setting. Moreover, CDK4/6 inhibitors are promising candidates for treatment of a subset of patients with tumors presenting CDK4 copy number gains and/or overexpression, while linsitinib might be an interesting combination partner in patients with both IGF2 and IGF1R overexpression.

These results are intended as a basis for a validation study in a prospective cohort, further evaluation *in vivo* in suitable mouse models or testing in patients with ACC in clinical trials are needed and might improve the future management of patients with ACC in terms of precision medicine.

Nebennierenrindenkarzinome (ACC) sind aggressive Tumore, die mit einer heterogenen, aber insgesamt ungünstigen Prognose sowie limitierten therapeutischen Optionen für fortgeschrittene Stadien assoziiert sind. Trotz hoffnungsvoll stimmenden molekularen Einblicken und verbessertem Verständnis für die Biologie des ACC wurden bisher keine effektiven Targeted Therapies (zielgerichtete Therapien) identifiziert. Daher strebt diese Studie die Identifikation potentieller neuer Drug Targets (Arzneimittelzielpunkte) im Rahmen einer zukünftigen personalisierten Therapie an.
RNA wurde von 104 formalinfixierten und paraffineingebetteten Tumorproben von ACC Patienten isoliert, 40 der 104 Fälle zeigten sich nach der Qualitätsprüfung der extrahierten RNA geeignet für weiterführende mRNA-Analysen. Genexpression von 84 bekannten Karzinom-Drug Targets wurden durch quantitative Real-Time PCR unter Nutzung von 5 normalen Nebennieren als Referenz evaluiert. Proteinexpression wurde in selektierten Kandidaten-Drug Targets durch Immunhistochemie in 104 ACC-Proben, 11 Adenomen und 6 normalen Nebennieren untersucht. Das Potential eines verfügbaren Inhibitors gegen das vielversprechendste Kandidatengen wurde in funktionalen *in vitro* Experimenten mit zwei ACC-Zelllinien (NCI-H295R und MUC1) allein und in Kombination mit einem anderen Medikament getestet.
Die am häufigsten überexprimierten Gene stellten TOP2A, IGF2, CDK1, CDK4, PLK4 und PLK1 dar. Die immunhistologische Kernfärbung für CDK1, CDK4 und PLK1 korrelierten signifikant mit der jeweiligen mRNA-Expression. CDK4 wurde als erfolgversprechendster Kandidat für weitere funktionale Validierung ausgewählt, da es durch FDA-genehmigte CDK4/6-Inhibitoren angreifbar ist. ACC-Proben mit Copy Number Gains des CDK4 Genlocus zeigten signifikant höhere CDK4 Expressionslevel. Der CDK4/6-Inhibitor Palbociclib wies eine zeit- und konzentrationsabhängige Reduktion der Zellviabilität *in vitro* auf, welche ausgeprägter in NCI-H295R- als in MUC1-Zellen war. Dies war in Einklang mit stärkerer CDK4 Expression in den NCI-H295R-Zellen in der Western Blot Analyse. Weiterhin wurde Palbociclib in Kombination

mit dem dualen IGFR/IR-Inhibitor Linsitinib eingesetzt, dies zeigte einen synergistischen Effekt auf die Reduktion der Zellviabilität.

Zusammenfassend bestätigte diese Proof-of-Principle den Nutzen von RNA Profiling zur Erfassung potentieller Drug Targets. Die ermittelten Drug Targets sind geeignet für immunhistochemische Untersuchungen im klinischen Setting. Darüber hinaus sind CDK4/6-Inhibitoren vielversprechende Kandidaten für die Behandlung einer Teilgruppe von Patienten mit Tumoren, die CDK4-Copy Number Gains und/oder -Überexpression aufweisen, während Linsitinib ein interessanter Kombinationspartner in Patienten mit sowohl IGF2- wie auch IGF1R-Überexpression darstellen könnte.

Diese Resultate sollen als Basis für eine Validationsstudie in einer prospektiven Kohorte dienen, weitere Evaluation *in vivo* in geeigneten Mausmodellen oder Untersuchung in ACC-Patienten in klinischen Studien sind erforderlich und könnten das zukünftige Management von ACC-Patienten verbessern im Rahmen der Präzisionsmedizin.

6. Bibliography

Several selected aspects of this thesis are presented as an abbreviated version in the publication (Liang et al., 2020), which was published during this thesis.

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7. Index

7.1. List of abbreviations

ACA	adrenocortical adenoma
ACC	adrenocortical carcinoma
ACTH	adrenocorticotropin
AKT	protein kinase B
APC	adenomatous polyposis coli
ATM	ataxia telangiectasia mutated
AURK	aurora kinase
BCA	bicinchoninic acid
BRCA	breast cancer type
BSA	bovine serum albumin
BUB1B	BUB1 mitotic checkpoint serine/threonine kinase B
CCND1	cyclin D1
CDC25A	cell division cycle 25A
CDK	cyclin-dependent kinase
CDKN2A	cyclin-dependent kinase inhibitor 2A
cDNA	complementary DNA
[^{11}C]MTO	metomidate
CN	copy number
CT	computerized tomography
CTNNB1	catenin beta 1
CYP	cytochrome P450
DAB	3,3'-diaminobenzidine
DAXX	death domain associated protein
DHEA	dehydroepiandrosterone
DHEA-S	dehydroepiandrosterone sulfate
DMEM	Dulbecco's modified Eagle medium
DMSO	dimethylsulfoxid
DNA	deoxyribonucleic acid
DPBS	Dulbecco's phosphate buffered saline

ECL	enhanced chemiluminescent
EDP-M	etoposide, doxorubicin, cisplatin and mitotane
EDTA	ethylenediamine tetraacetic acid
EGFR	human epidermal growth factor receptor
EMA	European Medical Association
ENSAT	European Network for the Study of Adrenal Tumors
ERCC1	excision repair cross-complementation group 1
ESE	European Society of Endocrinology
FC	fold change
FCS	fetal calf serum
FDA	U.S. Food and Drug Association
FDG-PET	fluorodeoxyglucose-positron emission tomography
FFPE	formalin-fixed paraffin embedded
FGFR	fibroblast growth factor receptor
FIRM-ACT	First International Randomised trial in locally advanced and Metastatic Adrenocortical Carcinoma Treatment
FLT1	fms-related tyrosine kinase 1
HDAC	histone deacetylase
HEK	human embryonic kidney
hENT1	human equilibrative nucleoside transporter type 1
HER2	human epidermal growth factor receptor 2
HRP	horseradish peroxidase
HU	Hounsfield unit
HR	hormone receptor
IGF	insulin growth factor
IGF1R	insulin growth factor 1 receptor
IHC	immunohistochemistry
IL-13-PE	interleukin-13 fused with a truncated form of *Pseudomonas* exotoxin A
IL-13Rα2	interleukin-13 receptor subunit alpha-2
[^{123}I]IMTO	iodometomidate
IR	insulin receptor

KIT	tyrosine-protein kinase kit
MED12	mediator complex subunit 12
MEN1 syndrome	menin 1, multiple endocrine neoplasia type 1
MRI	magnetic resonance imaging
mRNA	messenger RNA
mTOR	mechanistic target of rapamycin
mTORC1	mTOR complex 1
NAG	normal adrenal gland
NCI	National Cancer Institute
NF1	neurofibromin 1
OS	overall survival
PARP1	poly(ADP-ribose) polymerase 1
PD-1	programmed cell death protein 1
PD-L1	programmed death ligand 1
PI3K	phosphatidylinositol-3-kinase
PINK1	phosphate and tensin homolog induced kinase 1
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDGFR	platelet-derived growth factor receptor
PFS	progression-free survival
PLK	polo-like kinase
PR	partial response
PRKAR1A	protein kinase CAMP-dependent type I regulatory subunit alpha
PRKCA	protein kinase C alpha
RECIST	response evaluation criteria in solid tumors
RB	retinoblastoma
RBL	RB-like
RFA	radiofrequency ablation
RIPA	radioimmunoprecipitation assay buffer
RNA	ribonucleic acid
rpm	revolutions per minute
RRM1	ribonucleotide reductase catalytic subunit M1

RT	room temperature
RT-qPCR	quantitative reverse transcription PCR
SD	stable disease
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
SF	splicing factor 1
siRNA	small interfering RNA
SOAT1	sterol O-acyltransferase 1
TACE	transarterial chemoembolization
TBS-T	tris-buffered saline-Tween 20
TERT	telomerase reverse transcriptase
TOP2A	topoisomerase 2 alpha
TP53	tumor protein p53
Tris	tris(hydroxymethyl)aminomethane
TS	thymidylate synthase
TTP	time to progression
VEGF	vascular endothelial growth factor
VEGFR	vascular endothelial growth factor receptor
WB	western blot
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8. Appendix

Acknowledgement

First and foremost, I would like to offer my deepest thanks and appreciation to my supervisor and mentor PD Dr. Cristina Ronchi for her continuous support, guidance and care throughout the doctoral thesis. Thank you for introducing me to the world of science and research, for your patience and encouragement, for sharing your tremendous knowledge and insight with me. Dear Cristina, this thesis would have not been possible without you.

I would also like to extend my sincere gratitude to Prof. Dr. Fassnacht who gave me the opportunity of joining his lab. His overall guidance and insightful input accompanied this project to what it is today. A special thanks to both of them for providing invaluable opportunities and challenges and broadening my horizon. Working under their supervision taught me many things, both on professional and personal level.

I would also like to thank the two other members of my thesis committee: Prof. Dr. Andreas Rosenwald and PD Dr. Simone Rost. Thank you for the kind support, the specialist advice and suggestions as well as sparing time out of your busy schedules.

Moreover, I want to thank the whole endocrinology lab team, particularly Sonja Steinhauer, for the warm welcome, the introduction to the lab and their help during and after my time in the lab.

My thanks also go to the Graduate School of Life Sciences for their support and well-structured scientific training.

Last but not least, I want to thank my dearest family and friends. Charel, my partner in crime, we made it – cheers to the years to come! Paula, my dearest ally and friend. And, of course, always and ever, thanks to you, my wonderful parents, Song and Baozhu. Thank you for your unlimited loving support, motivation and guidance. Thank you for providing me with a solid base to rely on and for teaching me how to fish. Thank you for everything and for so much more.

Affidavit

I hereby confirm that my thesis entitled *Identification of new drug targets in adrenocortical carcinoma through targeted mRNA analysis* is the result of my own work. I did not receive any help or support from commercial consultants. All sources and / or materials applied are listed and specified in the thesis.

Furthermore, I confirm that this thesis has not yet been submitted as part of another examination process neither in identical nor in similar form.

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List of publications

Publications:

Liang R, Weigand I, Lippert J, Kircher S, Altieri B, Steinhauer S, Hantel C, Rost S, Rosenwald A, Kroiss M, Fassnacht M, Sbiera S, Ronchi CL. Cyclin Dependent Kinase 4 as promising drug target in adrenocortical carcinoma. Front. Endocrinol. 11:219. doi: 10.3389/fendo.2020.00219

Lippert J, Appenzeller S, **Liang R**, Sbiera S, Kircher S, Altieri B, Nanda I, Weigand I, Gehrig A, Steinhauer S, Riemens RJM, Rosenwald A, Müller CR, Kroiss M, Rost S, Fassnacht M, Ronchi CL. Targeted Molecular Analysis in Adrenocortical Carcinomas: A Strategy Toward Improved Personalized Prognostication. J Clin Endocrinol Metab. 2018 Dec 1;103(12):4511-4523. doi: 10.1210/jc.2018-01348

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