Charakterisierung der pathogenetisch-relevanten Rolle von SF1 beim Nebennierenrindenkarzinom

Characterisation of the pathogenetic-relevant role of SF1 in adrenocortical carcinoma

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To My Family,
Friends,
Mother Nature

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Will mankind recognize in time that this fascinating world is worth being protected? Time has come for each and everyone to make the decision if we want to be part of the problem or part of the solution.

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1 Zusammenfassung

Tumore der Nebennieren stellen häufige Tumore dar, welche bei mindestens 3 % der Population über 50-Jähriger vorkommen. Im Gegensatz dazu ist das Nebennierenrindenkarzinom mit einer Inzidenz von 1–2 Einwohner pro Million ein sehr seltener Tumor. Da seine Prognose allerdings ungünstig, und diese maßgeblich davon abhängt wie fortgeschritten der Tumor bei Diagnosestellung ist, ist es wichtig, dass die richtige Diagnose frühzeitig gestellt wird.

Bis heute ist kein zuverlässiger immunhistochemischer Nebennierenrindenkarzinom-spezifischer Marker etabliert um das Nebennierenrindenkarzinom von anderen retroperitonealen Tumoren zu differenzieren. Sasano et al. schlug bereits 1995 erstmalig den Transkriptionsfaktor Steroidogenic Factor 1 (SF1) als Marker zur Differenzierung von Nebennierenrinden- und Nicht-Nebennierenrindentumoren vor. Allerdings wurde die diagnostische Wertigkeit bisher nur in sehr kleinen Fallserien mit insgesamt nur 17 Nebennierenrindenkarzinomen untersucht.

In der vorliegenden Arbeit wurde die SF1 Protein-Expression bei 163 Nebennierenrindenkarzinomen, 52 Nebennierenrinden-Adenomen, 12 normalen steroidogenen Geweben (6 Nebennieren und 6 Ovare), sowie 73 Nicht-Steroidtumoren immunhistochemisch untersucht. Hierbei zeigte sich, das SF1 bei 158 von 161 evaluierbaren Nebennierenrindenkarzinomen und bei allen Proben von normalen und gutartigen Geweben (n=64) nachweisbar war. Im Gegensatz dazu war keine der 73 Nicht-Steroidgeweben SF1 positiv, so dass die diagnostische Genauigkeit extrem gut ist (Sensitivität: 98.6 %, Spezifität: 100 %, positive und negative predictive value jeweils 100 % und 97.3 %). In einem zweiten Schritt wurde untersucht ob die Protein-Expression von SF1 beim Nebennierenrindenkarzinom auch prognostische Bedeutung hat. Hierbei zeigte sich, dass Patienten mit Tumoren mit starker SF1 Färbung (30 %) ein deutlich schlechteres tumorstadium-adjustiertes Rezidiffreies- und Gesamt-Überleben haben als Patienten mit geringer SF1 Expression (hazard ratio: 2.45). Zusätzlich zu den immunhistochemischen Untersuchungen wurden FISH Analysen durchgeführt. Hierbei zeigte sich allerdings keine signifikante Korrelation zwischen *SF1* Gendosis und der SF1 Protein-Expression, so dass zu vermuten ist, dass *SF1* maßgeblich auf Transkriptions- und Translationsebene reguliert wird. In einem Versuch diese Frage zu beantworten wurden zwei mutmaßliche SF1 Interaktionspartner, FATE1 und DAX1, genauer immunhistochemisch untersucht. Hierbei wurde deutlich, dass FATE1 bei 62 von 141 evaluierbaren Nebenierenrindenkarzinomen und 12 von 62 normalen und gutartigen Geweben nachweisbar war. Im Gegensatz hierzu waren alle 9 Nicht-Steroidgewebe FATE1 negativ. Dies zeigt, das FATE1 nicht zur Diagnostik nutzbar ist (Sensitivität: 61 %, Spezifität: 100 %, positive und negative predictive value 100 % bzw. 14 %). Die DAX1 Analyse zeigte, dass alle 20 normalen und gutartigen Gewebe eine positive DAX1 Färbereaktion zeigten. Von 126 Nebennierenrindenkarzinomen waren 71 DAX1 positiv. Von den 8 untersuchten Nicht-Steroidgeweben waren 6 DAX1 positiv. Diese Ergebnisse belegen, dass auch DAX1 keine diagnostische Genauigkeit besitzt (Sensitivität: 56 %,

Spezifität: 25 %, positive und negative predictive value 92 % bzw. 4 %). Die Untersuchung der prognostischen Fähigkeiten von FATE1 und DAX1 zeigte, dass Patienten mit Tumoren mit starker FATE1 Färbung (39 %) ein schlechteres tumorstadium-adjustiertes Gesamt- aber nicht Rezidiffreies-Überleben haben als Patienten mit niedriger FATE1 Protein-Expression (hazard ratio: 2.01). Weiterhin wurde deutlich, dass DAX1 keine deutlichen prognostischen Fähigkeiten besitzt.

Zusammenfassend läßt sich aus der vorliegenden Arbeit folgern, das SF1 aktuell der beste diagnostische Marker zur Diagnose von Tumoren der Nebennierenrinde ist und damit Eingang in die histopathologische Routine-Diagnostik von Nebennierentumoren finden wird. Zusätzlich ist die SF1 Expression ein sehr guter prognostischer Marker beim Nebennierenrindenkarzinom, wobei sich die prognostische Aussage durch zusätzliche Färbung von FATE1 und DAX1 nur unwesentlich verbessern läßt.

2 Abstract

Adrenal tumors are common tumors which are present in at least 3 % in the human population over their 5th decade. However, adrenocortical carcinoma (ACC) is a rare malignancy which shows an approximate anual incidence of 1–2 per million. Prognosis of ACC is generally poor and depends strongly on the tumor stage. Thus, early and correct diagnosis is important.

Until now, no reliable immunohistochemical ACC-specific marker has been established for its differentiation from other retroperitoneal tumors. Already in 1995, Sasano et al. suggested the transcription factor Steroidogenic Factor 1 (SF1) as useful marker for differentiation of adrenocortical and non-adrenocortical tumors. Up to now, SF1's value as diagnostic marker for ACC was investigated only in small series of in a total of 17 samples. In our work, SF1 expression was investigated by immunohistochemistry in 163 ACC, 52 adrenocortical adenomas, 12 normal steroidogenic tissues (6 adrenal glands and 6 ovaries), as well as 73 non-steroidogenic tumors. SF1 protein expression was shown in 158 of a total of 161 evaluable ACC, as well as all normal and benign steroidogenic tissues. In contrast, no SF1 protein was detectable in the non-steroidogenic tumors. Thus, SF1 protein expression is a highly specific diagnostic tool (sensitivity: 98.6 %, specificity: 100 %, positive and negative predictive value: 100 % and 97.3 %, respectively). In a second step, SF1 protein expression was investigated as a prognostic tool in ACC. As shown by us, ACCs presenting strong SF1 immunoreactivity (30 %) showed a strong correlation with overall and recurrence-free patients survival than ACCs presenting low SF1 protein expression (hazard ratio: 2.45). Moreover, FISH analyses were performed which revealed no significant correlation of *SF1* gene dosis and SF1 protein expression, suggesting a regulatory mechanism at transcriptional and translational level. To investigate the hypothesis, we investigated two putative interaction partners of SF1, namely FATE1 and DAX1 protein, by immunohistochemistry. FATE1 protein was expressed in 62 of a total of 141 evaluable ACC as well as 12 of a total of 62 normal and benign steroidogenic tissues. In contrast, all non-steroidogenic tissues were FATE1 negative (n=9). Thus, FATE1 is no valuable diagnostic tool (sensitivity: 61 %, specificity: 100 %, positive and negative predictive value: 100 % and 14 %, respectively). DAX1 immunohistochemistry showed that all normal and benign steroidogenic tissues (n=20) were DAX1 positive as well as 71 of a total of 126 ACC samples. Furthermore, 6 out of a total of 8 non-steroidogenic tissues stained DAX1 positive, showing that DAX1 protein is no diagnostic tool (sensitivity: 50 %, specificity: 25 %, positive and negative predictive value: 92 % and 4 %, respectively). Investigation of the prognostic value of FATE1 and DAX1 revealed that patients with tumors characterized by strong FATE1 immunoreactivity (39 %) had a worse outcome in overall but not recurrence-free survival than patients showing low FATE1 expression (hazard ratio: 2.01). DAX1 protein expression has no prognostic value in ACC.

In summary, we showed that SF1 is currently the best available diagnostic marker for differentiation of adrenocortical tumors from other retroperitoneal tumors, and that it will be suitable for histopathological diagnostic routine. Furthermore, SF1 expression is a well-suited prognostical tool in adrenocortical carcinoma which is only marginally enhanced by subsequent staining of FATE1 and DAX1 protein.

Docendo discimus.[a]

Seneca, epistulae morales 7,8

[a]By teaching, we learn.

3 Introduction

3.1 The adrenal cortex

The adrenal cortex is an integral part of both adrenal glands (*glandulae suprarenales*) which are located at the cranial part of the kidneys and show an approximate weight of 4–5 g. The adrenal glands were first described by Eustachi [1574] and already in 1855 Addison indicated their necessity for survival [Addison, 1855]. The adrenal cortex is important for the endocrine activity of adrenal glands and is located between the capsule and *medulla*.

3.1.1 Development of adrenal glands

Human adrenal cortices arise from a combined adrenal and gonadal precursor (adrenogonadal primordium) at 4 weeks of gestation; coelomic and/or the underlying mesonephric mesenchymal cells condensate between the dorsal mesentary and urogenital ridge [Sucheston and Cannon, 1968, Mesiano and Jaffe, 1997]. Hereafter, cells migrate to the cranial pole of the *mesonephros* in two waves to form the adrenal primordium. The adrenal primordium presents as a homogenous cell aggregation from which the fetal and definitve zones arise (see below).

The inner part of the adrenal gland is the *medulla*, which develops from neural crest-derived chromaffin cells that migrate to the adrenal primordium [Keegan and Hammer, 2002]. Thus, its origin is rooted in neural cells which lack steroidogenic capacity.

The fetal adrenal cortex

The human fetal adrenal cortex shows two morphologically distinct zones. The outer definitve/-adult zone is composed of small, tightly packed cells that is first steroidogenically silent (they do not express key enzymes of steroidogenesis, i.e. cytochrome P450, family 17, subfamily A (CYP17A) [Hanley et al., 2001, Narasaka et al., 2001]). However, late in gestation it acquires steroidogenic capabilities due to expression of enzymes involved in steroidogenesis; consequently the definitive zone is thought to be comparable to adult *zona glomerulosa* [Coulter and Jaffe, 1998]. The inner fetal zone is steroidogenically active (it expresses CYP17; [Hanley et al., 2001, Narasaka et al., 2001]). The primary secreted steroid of the fetal zone is dehydroepiandrosterone sulfate (DHEAS). Thus, the fetal zone shows characteristics of the adult *zona reticularis* [Coulter and Jaffe, 1998].

The fetal zone consists of large, lipid-containing cells comprising approximately 85–90 % of the fetal adrenal gland and degenerates primarily due to apoptosis after birth within the first year of life [Bech et al., 1969, Mesiano and Jaffe, 1997]. Ehrhart-Bornstein et al. [1998] suggest that cytokines produced by the inner zones of human adrenal cortices might participitate in the differentiation and apoptosis of *zona reticularis*.

It is believed that the fetal zone as well as parts of adult adrenal zones derive from precursors of the definitive zone [Mesiano and Jaffe, 1997, Keegan and Hammer, 2002, Muench et al., 2003]; it is suggested that this zone containes a progenitor cell population [Wolkersdoerfer and Bornstein, 1998].

In humans, between the outer definitve zone and fetal zone a third zone (transition zone) is located that is capable of glucocorticoid production and secretion around third trimester [Mesiano et al., 1993, Parker et al., 1995, Coulter et al., 1996, Mesiano and Jaffe, 1997]. Thus, the transitional zone has steroidogenic capacities comparable to *zona fasciculata* of the adult [Coulter and Jaffe, 1998].

Disruption of the hypothalamo-pituitary axis in the human fetus results in abrogation of growth of the fetal zone after 16 weeks of gestation.

Substantial evidence (reviewed by Mesiano and Jaffe [1997], Coulter [2004]) indicates that adrenocorticotrophic hormone (ACTH) influences fetal adrenal growth *in vivo* by intra-adrenal growth factors, e.g. insuline-like growth factor II (IGF-II), basic fibroblast growth factor (bFGF) and epidermal growth factor (EGF). IGF-II protein levels are highest in blood circulation and tissue of fetal life, but decreases postnatally [D'Ercole, 1991]. However, a total of 69 genes are thought to be involved in the development and growth of fetal adrenal glands [Rainey et al., 2001].

3.1.2 Adult adrenal cortex

The adult adrenal cortex can be histologically divided into three zones that are encapsulated in connective tissue (*textus connectivus*). *Zona glomerulosa* is the peripheral zone which synthesizes and secretes primarily mineralcorticoids (mainly aldosterone). Cells of this zone are relatively small, agglomerated and strand-like aligned. The next zone is *zona fasciculata*. Its cells are grouped in strands and radially arranged. The main (in sini) secreted steroids are glucocorticoids (mainly cortisol). From all three zones, the cell diameter of cells of *zona fasciculata* is largest. The most inner zone is *zona reticularis*. This zone secretes primary dehydroepiandrosterone (DHEA) but also glucocorticoids. Cells of *zona reticularis* show a net-like structure. After this zone the *medulla* follows. Figure 3.1 shows the build of an exemplary adrenal cortex.

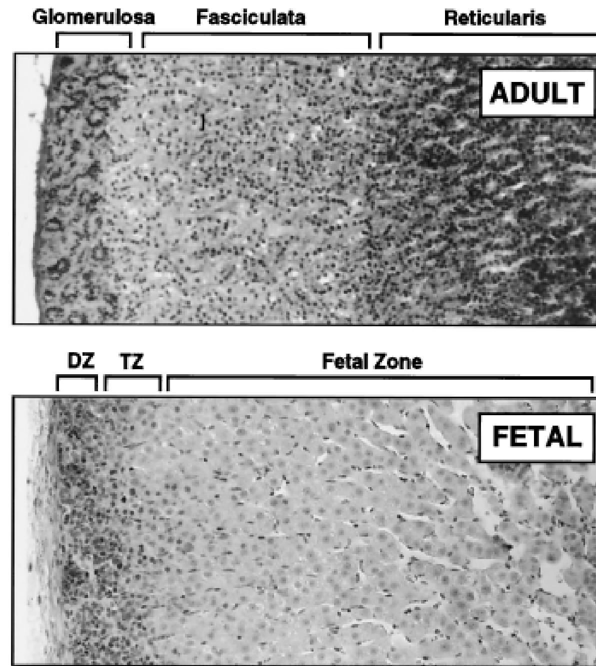

Figure 3.1: Exemplary build of an adrenal cortex. Top: zonation of an adult adrenal. Capsule and *zonae glomerulosa, fasciculata*, and *reticularis* are shown. Bottom: zonation of fetal adrenal gland. DZ: definitive zone. TZ: transition zone. Note the small, tightly packed cells of the definitive zone and the larger and irregular aligned cells of the fetal zone. Picture taken from Else and Hammer [2005].

3.2 Steroidogenic Factor 1 protein

3.2.1 Overview of SF1

The SF1 protein (also known as NR5A1 and Ad4BP) belongs to orphan nuclear receptors, since, until now, no 'real' ligand has been identified. The *SF1* gene was isolated 1992 independently by two groups and shown to encode for an approximately 53 kilodalton (kDa) protein [Morohashi et al., 1992, Lala et al., 1992] of 462 amino acids. Competition experiments revealed that it binds not only to its originally identified recognition sequence (C/T)CAAGG(T/C)(C/T), but also to (Pu)PuPuAGGTCA; nonetheless, (C/T)CAAGG(T/-C)CA presents the strongest binding sequence [Morohashi et al., 1992]. Taketo et al. [1995] mapped the gene to chromosome 9q33 using fluorescence *in situ* hybridization. Figure 3.2 shows the localization of *SF1* on chromosome 9.

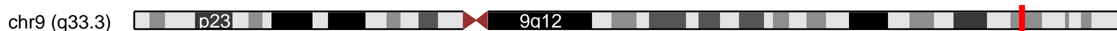

Figure 3.2: Localization of *SF1* on chromosome 9. The red bar indicates the approximate position of the gene *NR5A1* at 9q33. Figure extracted from `http://genome.ucsc.edu/` in June, 2010.

The promoter of *SF1* is subject to methylation in that hypomethylation occurs in cells and tissues which express *SF1* but is hypermethylated in cells and tissues that do not express *SF1* [Xue et al., 2007, Hoivik et al., 2008]. *SF1* mRNA (3.5 kb) is found mostly in normal steroidogenic tissues like the adrenal cortex, ovary, and testis.

Ueda et al. [1992] showed that SF1 acts as a monomer. On the search for SF1-specific ligands it was shown that oxysterols are not able to activate the protein in most cell lines [Lala et al., 1997, Mellon and Bair, 1998, Christenson et al., 1998]. In 2005, phospholipids were shown to be bound in the ligand binding pocket of crystalized SF1 [Krylova et al., 2005, Li et al., 2005, Wang et al., 2005]. Figure 3.3 shows the ligand binding domain (LBD) of NR5A1 bound to phosphatidylcholine and a phospholipid comprised of phosphatidylethanolamine and glycerol moieties [Li et al., 2005, Sablin et al., 2009].

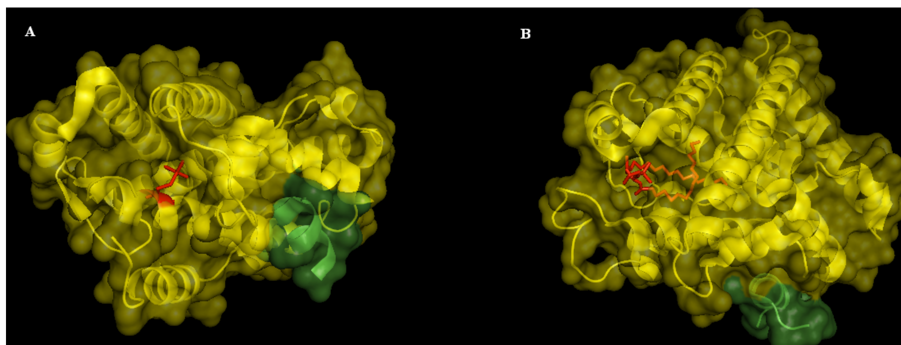

Figure 3.3: Structure of SF1's ligand binding domain bound to phospholipids. A: ligand binding domain of SF1 (yellow) with bound phosphatidylcholine (red). Green: peptide of PPAR-γ. B: ligand binding domain of SF1 (yellow) with bound phospholipid comprised of phosphatidylethanolamine and glycerol moieties (red). Green: peptide of SHP. Under each translucent surface the corresponding amino acid sequence is shown in cartoon presentation. PDB files used: 3F7D [Sablin et al., 2009] (A) and 1YP0 [Li et al., 2005] (B).

SF1 is capable of forming a specific complex with DNA through a bipartite motif which binds to the major and minor grooves of the nucleic acid through the core DBD and the N-terminal segment of the FtzF1 box, respectively (Figure 3.4) [Little et al., 2006]. Furthermore, the FtzF1 box is suggested to be an important servant of stability for the complex, as well as serving as an interaction platform with coactivators and other DNA-binding factors in the vicinity [Little et al., 2006].

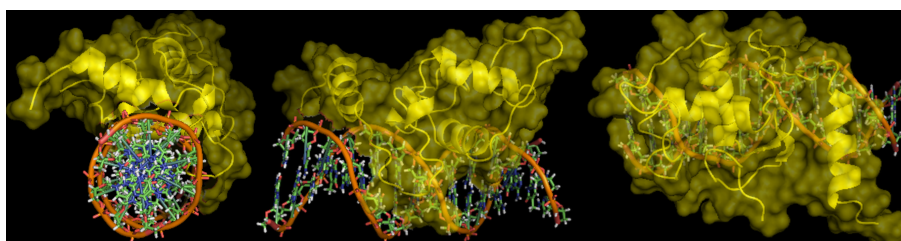

Figure 3.4: DNA binding by SF1's DNA binding domain. Different views of DNA bound by SF1s' DNA binding domain (yellow) are shown. Left to right: front, side and top view. Under each translucent surface the corresponding amino acid sequence is shown in cartoon presentation as well as the deoxyribonucleic acid double helix. PDB file used: 2FF0 [Little et al., 2006].

3.2.2 Interaction partners and posttranslational modifications of SF1

SF1 interacts with a couple of proteins and is also posttranslationally modified by some of them. A brief overview is given below.

Interaction partners of SF1 Interaction partners of the nuclear receptor Steroidogenic Factor 1 protein are cAMP response element binding protein (CREB) which leads to hormone regulated protein expression of the aromatase gene [Carlone and Richards, 1997], glucocorticoid receptor interacting protein 1 (GRIP1) resulting in enhancement of glucocorticoid receptor induction [Eggert et al., 1995], as well as transcription factor nuclear receptor coactivator 1/steroid receptor coactivator 1 (NCOA1/SRC1) reported to be components of the coactivator complex [Ito et al., 1998]. Furthermore, interactions of NCOA3 (pCIP/SRC3/ACTR/AIB1), a SF1 enhancer, [Borud et al., 2002] and the negative SF1-regulator DAX1 are described [Crawford et al., 1998, Nachtigal et al., 1998].

Acetylation of SF1 Among others, the histone acetyltransferases (HATs) K(lysine) acetyltransferase 2B (KAT2B/PCAF) and E1A binding protein p300 (EP300) are capable of acetylating SF1 [Chen et al., 2005, Jacob et al., 2001] increasing its activity. If the acetylation site KQQKK [Chen et al., 2005] and K34, K38, and K72 located in the N-terminal domain within the zinc finger motifs [Jacob et al., 2001] are mutated to R, acetylation of SF1 is prevented and its transcriptional activity decreases [Chen et al., 2005, Jacob et al., 2001]. Inhibition of acetylation by the HAT inhibitor trichostatin A (TSA) increases SF1's activity and half-live [Jacob et al., 2001, Tremblay et al., 2009]. Furthermore, SF1 acetylation by EP300 enhances its localization within transcriptionally active nuclear foci [Chen et al., 2005].

Phosphorylation of SF1 Protein phosphorylation by certain kinases serves as an ubiquitination signal followed by proteasome-mediated degradation of the target protein [Lange et al., 2000]. The ubiquitin-proteasome pathway is the major pathway for selective degradation of cellular proteins in eukaryotes [Pickart, 1997].

The primary structure of SF1 suggests a putative cAMP-dependent protein kinase (PKA) phosphorylation site at Ser^{430} [Honda et al., 1993]. Contrary to degradation, Aesoy et al. [2002] showed that PKA-mediated phosphorylation of SF1 increases protein stability but is not essential for *steroidogenic factor 1* expression or transcriptional activity.

Mitogen-activated protein kinase 1 (MAPK1; also known as extracellular signal-regulated kinase 2 [ERK2]) is capable of phosphorylating SF1 *in vitro* at Ser^{203} which is located in the AF1 domain within a PYASP motif that resembles the PX_nS/TP consensus site of serine/threonine MAPKs [Hammer et al., 1999, Gonzalez et al., 1991]. Furthermore, phosphorylation of Ser^{203} modulates the activity of SF1 in the absence of ligand due to enhanced cofactor binding to the LBD and is critical for maximal activation, mediated by components of the MAPK signaling pathway [Hammer et al., 1999]. Moreover, Lewis et al. [2008] showed that cyclin-dependent kinase 7 (CDK7), a constituent of the basal transcription factor TFIIH, also phosphorylates SF1 at Ser^{203} *in vitro*. The CDK7-mediated phosphorylation of serine 203 "is coupled on the integrity of the LBD and transcriptional activation, indicating a correlation between ligand binding, phosphorylation status, and functionality" [Lewis et al., 2008].

SUMOylation of SF1 SUMOylation occures at the consensus sequence ψKxE, where ψ is a large hydrophobic residue, K the covalently SUMOylated lysine, x any amino acid and E

glutamic acid [Sampson et al., 2001]. The mechanism itself is enzyme-mediated and analogous to ubiquitination [Capili and Lima, 2007].

SF1 (and other NR5 receptors) can be SUMOylated at conserved IKSE or I/VKQE motifs in the Hinge region [Lee et al., 2005]. Ou et al. [2001] showed that the DEAD box protein DP103 directly interacts with the proximal repression domain (amino acids 193–201) of SF1 and represses its transcriptional activity. Recently, Lee et al. [2005] published evidence that for SUMO-mediated repression of SF1 activity DP103 interacts directly with SUMOylated SF1 and that repression was histone deacetylase independent and uninfluenced by the AF2 corepressor DAX1. Moreover, ATPase/RNA helicase DP103 enhanced protein inhibitors of activated STAT (PIAS)-dependent nuclear receptor SUMOylation (PIAS belongs to E3-SUMO ligases) and promotes PIASy-mediated transport of SF1 to discrete nuclear bodies/foci [Lee et al., 2005]. Three years later, it was shown that SUMOylation of K119 and K194 (which are proximal to the DBD and LBD, respectively) impaired the normal function of these domains [Campbell et al., 2008]. Furthermore, *i.)* K194 SUMOylation decreased modestly Ser^{203} phosphorylation but did not affected LBD structure, *ii.)* SUMOylation influenced strongly SUMOylated SF1 in that it was unable to bind noncanonical, atypical SF1 recognition sequences, and *iii.)* DNA-bound SF1 is refractory to SUMOylation which implies that it can not be posttranslationally modified by SUMOylating enzymes as long as it is bound to nucleic acid [Campbell et al., 2008]. Yang et al. [2009] showed that *i.)* SUMOylation represses target gene transcription but did not alter nuclear localization, *ii.)* SUMOylation is not altered by ACTH induction or SF1 phosphorylation but, instead, prevention of SUMOylation enhanced CDK7-mediated SF1 phosphorylation.

3.2.3 SF1 in transcriptional regulation and tumorigenesis

SF1 regulates steroidogenic as well as non-steroidogenic proteins like CYP17, DAX1, CYP19, CYP11A1, MIS, 3β-HSD, CYP21, StAR, Mc2R and FATE1 [Burris et al., 1995, Carlone and Richards, 1997, Kawabe et al., 1999, Kelly et al., 2004, Leers-Sucheta et al., 1997, Liu and Simpson, 1997, Sugawara et al., 1996, Watanabe et al., 2000, Winnay and Hammer, 2006, Zhang and Mellon, 1996, Doghman et al., 2007].

It was shown that SF1 plays a crucial role in the regulation of the human gene *POU class 5 homeobox 1* (*POU5F1*; also known as *octamer-4 [OCT4]*), a transcription factor involved in maintaining self-renewal in stem cells, presenting three putative SF1 binding sites [Yang et al., 2007]. It might be noteworthy that SF1 overexpression increased *POU5F1* promoter activity and endogenous POU5F1 protein expression [Yang et al., 2007].

Pianovski et al. [2006] showed that in southern Brazil, *SF1* is overexpressed in childhood adrenocortical tumors, combined with increased *SF1* gene copy number and amplification. No significant correlation was observed either between protein level and gene number or any of the clinical parameters examined, so that they concluded that not only gene number variation might be responsible for childhood adrenal tumors but also epigenetic mechanisms. *In vitro* and *in vivo* data support the tumorigenic role of SF1 since increased dosage of it *i.)* augments proliferation and decreases apoptosis rates of human adrenocortical cells, and *ii.)* elevated SF1 levels drive establishement of adrenocortical tumors in mice [Doghman et al., 2007]. Further support for the 'tumor-driver SF1' hypothesis comes from a study which showed that SF1 inverse agonists are capable of inhibiting growth of SF1-positive human adrenocortical carcinoma cell line NCI-H295R, although SF1-negative SW13 cells were also susceptible to growth inhibition [Doghman et al., 2009].

3.3 Fetal and adult testis expressed 1 protein

The *fetal and adult testis expressed 1* (*FATE1*; also known as *cancer/testis antigen 43* [*CT43*]) promoter presents a putative SF1 binding site at position −79 to −71 upstream of the transcriptional start site [Olesen et al., 2001]. Doghman et al. [2007] showed that increased SF1 levels increased its binding to the *FATE1* promoter followed by recruitment of specific cofactors.

FATE1 is localized on chromosome Xq28, encompassing 7 kb genomic DNA (consisting of 5 exons and 4 introns) that translate into a 183 amino acid protein showing a molecular weight of 21 kDa [Olesen et al., 2001]. Its expression is shown in normal testis and weakly in normal pancreas [Dong et al., 2003]. Olesen et al. [2001] showed that FATE1 is faintly expressed in adults in lung, heart, kidney, adrenal gland as well as whole brain. The FATE1 protein is thought to control cell proliferation since it is mainly detectable in moderate to poor differentiated hepatocellular carcinoma [Yang et al., 2005].

3.4 DAX 1 protein

3.4.1 Overview of DAX1

The gene *nuclear receptor subfamily 0, group B, member 1 (NR0B1)*, also known as *DAX1* (dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X, gene 1), maps to chromosome Xp21 and encodes the adrenal-expressed DAX1 protein which was isolated by Zanaria et al. [1994]. It is composed of two exons (1168 bp and 245 bp, respectively) seperated by an intron of 3385 bp length [Zanaria et al., 1994]. The protein itself consists of 470 amino acids, shows a ligand binding domain (LBD) but misses a DNA binding domain (DBD); instead the DBD is substituted by a N-terminal structure composed of four incomplete, R- and G-rich 65–67 amino acid motifs which contain conserved cysteine residues [Zanaria et al., 1994]. Mutations in *DAX1* cause X-linked adrenal hypolasia congenita and hypogonadotropic hypogonadism [Muscatelli et al., 1994, Guo et al., 1995, Zanaria et al., 1994]. Figure 3.5 shows two DAX1 proteins bound to one NR5A2 (liver receptor homolog 1 [LRH1]) protein.

It was shown that DAX1 is capable of trafficking between the nucleus and cytoplasm, associated with ribonucleoprotein structures in the former and with polyribosomes in the latter [Lehmann et al., 2002, Lalli et al., 2000].

DAX1 is highly expressed in hormonally inactive adrenal tumors but low in endocrine active adrenal tumors [Reincke et al., 1998]. Zwermann et al. [2005] showed that DAX1 is able to repress transcription of the *ACTHR* gene in adrenocortical tumors dependent of SF1 binding sites within the *ACTHR* promoter. Gummow et al. [2006] reported that glucocorticoids are capable to increase *DAX1* gene expression mediated by *DAX1* promoter bound glucocorticoid receptor and that ACTH induces its own and SF1 promoter clearance.

In 2004 two groups published evidence that an alternatively spliced form of DAX1 exists, named DAX1-α/DAX1A, composed of exon 1 and exon 2A (showing a length of 35 nt and located in the 3385 bp intron) [Ho et al., 2004, Hossain et al., 2004] which, interestingly, differ by a single nucleotide polymorphism. The protein DAX1A measures approximately 400 amino acids in length [Ho et al., 2004, Hossain et al., 2004].

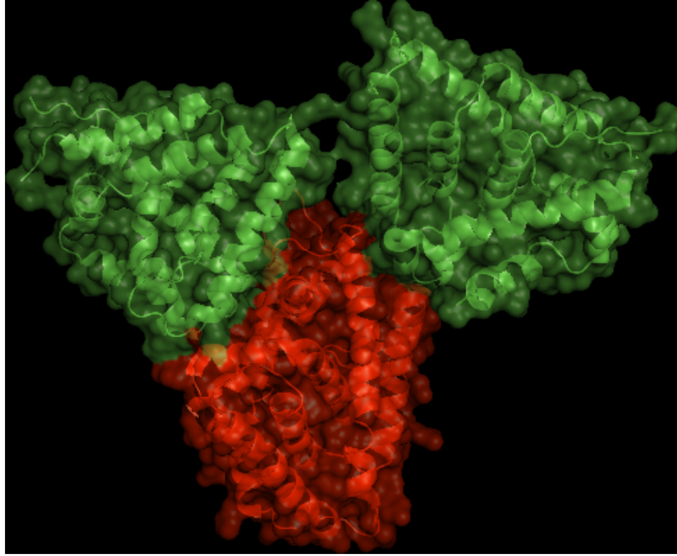

Figure 3.5: Two DAX1 proteins (green) bound to one NR5A2 protein (red). Under each translucent surface the corresponding amino acid sequence is shown in cartoon presentation. PDB file used: 3F5C [Sablin et al., 2008].

In 2007 it was shown that DAX1-α is mostly cytoplasmic localized whereas DAX1:DAX1-α heterodimers show a more nuclear distributional prevalence [Iyer et al., 2007]. Nakamura et al. [2009a] showed that *DAX1* mRNA level was significantly higher than *DAX1-*α mRNA levels in, among others, human fetal and adult adrenals, testis, and ovary; moreover, *DAX1-*α mRNA was undetectable in human liver, placenta, and kidney. Hossain et al. [2004] reported *DAX1-*α mRNA in the fetal kidney and in contrast to Nakamura et al. [2009a] as highest in human adrenal glands, testis, and ovary.

It might be difficult to differentiate both DAX1 isoforms since some commercially available DAX1-specific antibodies are raised against exon 1 (e. g. K-17 and H-300 from Santa Cruz Biotechnology, and 2F4 and 2E5 from the Sassone-Corsi laboratory) [Ho et al., 2004], so that for discrimination of both isoforms antibodies against carboxy-terminal sequences may be used.

3.4.2 Role of DAX1 in regulation of SF1 and *vice versa*

The carboxy-terminal transrepression domain of DAX1 is implicated in SF1-mediated transrepression [Ito et al., 1997, Sandhoff and McLean, 1999]. Interestingly, the C-terminal transrepression domain is deleted in most naturally occuring DAX1 deletion mutations [Ito et al., 1997].

Whether the C-terminal DAX1 transrepression domain is neccessary for DAX1:SF1 interaction, is controversial [Ito et al., 1997, Nachtigal et al., 1998, Lopez et al., 2001]. Furthermore, Hossain et al. [2004] reported that the DAX1 isoform DAX1-α is capable to interact directly with SF1 although it is unable to repress SF1-mediated promoter activation and serves as a DAX1 antagonist in special circumstances.

On the other hand, Mizusaki et al. [2003] showed that *DAX1* gene transcription is activated by CTNNB (β-catenin) in synergy with SF1. Furthermore, it was shown that synergy between SF1 and glucocorticoid receptor activates *DAX1* in a dose- and glucocorticoid-dependent

fashion, which is antagonized by ACTH signaling [Gummow et al., 2006]. ACTH-mediated down regulation of DAX1 by subsequent SF1 protein accumulation was confirmed by Ragazzon et al. [2006].

Interestingly, a recently published DAX1 investigation revealed that it might function as a negative regulator of *OCT4* to maintain proper OCT4 activity in mouse embryonic stem cells [Sun et al., 2009]. In contrast, *DAX1* is very low expressed in human embryonic stem cells and shows an inconsistent expression pattern during their differentiation [Xie et al., 2009]. Oda et al. [2009] found that in lung adenocarcinoma higher expression of *DAX1* correlated with higher tumor recurrence and higher rates of lymph node metastases which was inversely correlated to the proportion of the CpG methylation of the numerous CpG sites found around the DAX1 promoter.

DAX1 shows a putative SF1 responsive element which is specifically bound by SF1 [Burris et al., 1995, Vilain et al., 1997]. Moreover, SF1 enhances the transcriptional activity of the *DAX1* promoter by binding to its responsive element [Vilain et al., 1997].

3.5 Adrenal tumors

Adrenocortical tumors (ACT) are common tumors which present at least 3 % in a population above the 5th decade [Grumbach et al., 2003]. They can be differentiated into different tumor entities that are introduced briefly below.

3.5.1 Adrenocortical adenoma

Adrenocortical adenomas are monoclonal [Abrams et al., 1950], benign tumors of the adrenal cortex. They can be differentiated into aldosterone-producing Conn-, cortisol-producing Cushing-, and hormonally inactive- adenomas, showing an approximate frequency of 8 %, 17 %, and 2 % in a series of surgically removed adrenal neoplasms [Saeger, 1995]. Cushing adenoma shows enlarged and lipid-containing cells that are widespread among more common compact cells, whereas cells of Conn adenomas are frequently observed mainly large, clear cells rich of lipids and similiar to cells of the *zona fasciculata* [Sommers, 1977]. However, discrimination of adrenocortical adenoma from adrenocortical carcinoma is difficult (see section 3.6.4 for an explanation).

3.5.2 Pheochromozytoma

Pheochromocytoma is the most frequent tumor of the adrenal *medulla* [Marx et al., 1996]. The tumor cells resemble normal pheochromocytes [Saeger, 2000]. Saeger [2000] stated that approximately 10 % of all pheochromocytoma show malignant behaviour. If metastases are absent, no definite differentiation between malignant and benign pheochromocytoma is possible [Saeger, 2000]. Diagnosis of pheochromocytoma might be challenging if catecholamins are secreted in low concentrations and the plasma level is only marginally elevated — which can also be characteristics of an endocrine-inactive adrenocortical tumor. Thus, there is a certain risk to diagnose an endocrine-inactive adrenocortical tumor instead of pheochromocytoma [Saeger, 1995].

3.5.3 Metastases of tumors to the adrenal gland

The adrenal gland is reported as the fourth most common site of metastasis in humans — devastated by malignant neoplasias like melanoma, breast, lung, renal, and gastrointestinal cancer [Abrams et al., 1950, Lloyd et al., 2004]. From all patients that die due to cancer, approximately 19–27 % of patients present metastases in one adrenal, 56 % in both [Bodie et al., 1989, McNicol et al., 1997]. The metastases are found almost exclusive in *zonae fasciculata* and *reticularis* [Bodie et al., 1989]. Thus, if metastasis from other (non-steroidogenic) tumors arise in the adrenal gland, their differentiation from endocrine-inactive adrenocortical tumors is challenging.

3.5.4 Other adrenal tumors

Other tumors of the adrenal gland can be, e.g. myelolipoma, lipoma, adenomatoid tumors, and tumor-like lesions like cysts. Lipomatous tumors, like lipoma and myeolipoma, are often benign and endocrine-inactive tumors [Lack, 1997, Lesbats-Jacquot et al., 2007]. Myeolipomas have to be differentiated from adrenocortical adenomas since the latter may present extensive myelolipomatous areas (adenomas with myelolipomatous metaplasia) [Lack, 1997]. From the non-neoplastic cysts (endothelial, hemorrhagic/pseudocystic, parasitic, and epithelial cysts), differentiation of a pseudocyst from adrenocortical adenoma or carcinoma might be difficult if cortical cell islands surrounded by thrombus are present [Gaffey et al., 1989].

3.6 Adrenocortical carcinoma

3.6.1 General overview about adrenocortical carcinoma

Adrenocortical carcinoma (ACC) is a rare malignancy which shows an approximate incidence of 1–2 per million, characterized by a general poor prognosis and heterogenous presentation [Wajchenberg et al., 2000, Dackiw et al., 2001]. ACC comprised 0.2 % of deaths from cancer in one large series [Steiner, 1954]. Women seem to suffer more frequently from ACC than men with a ratio of 1.5 [Luton et al., 1990, Wooten and King, 1993, Icard et al., 2001, Koschker et al., 2006]. Age distribution is bimodal encompassing childhood and 4th–5th decade [Luton et al., 1990, Wajchenberg et al., 2000]. An unexpected, high incidence of ACC is reported in children in southern Brazil which seems to be correlated to mutations within the tumor repressor gene *TP53* [Ribeiro et al., 2001, Michalkiewicz et al., 2004]. The main organs/tissues to which ACC metastasizes are lung, liver and lymph nodes [Hutter and Kayhoe, 1966, Hajjar et al., 1975]. Less frequently, it metastasizes to bone and kidney [Hutter and Kayhoe, 1966, Hajjar et al., 1975]. In contrast to Lipsett et al. [1963], Hutter and Kayhoe [1966] and Hajjar et al. [1975] found also brain metastases.

Overall 5-year survival of ACC patients ranged in varying series between 16–44 % [Venkatesh et al., 1989, Luton et al., 1990, Pommier and Brennan, 1992, Soreide et al., 1992, Wooten and King, 1993, Haak et al., 1994, Bellantone et al., 1997, Schulick and Brennan, 1999b, Abiven et al., 2006]. In the German ACC registry 5-year and 10-year survival was 47 % and 41 %, respectively, and prognosis was associated with tumor stage [Fassnacht and Allolio, 2009]. 5-year survival was 84 % for stage I, 63 % for stage II, 51 % for stage III and 15 % for stage IV of the ENSAT staging system (see Table 3.1 on page 20) [Fassnacht and Allolio, 2009].

Sporadic adrenal tumors seem to be mainly of monoclonal origin although their molecular aetiology is still unknown [Beuschlein et al., 1994, Gicquel et al., 1994, Bernard et al., 2003]. It is also suggested that ACC evolves from adrenal adenomas after a second hit paradigm [Bernard et al., 2003, Cofield et al., 2005]. However, this hypothesis could never be proven.

ACC is further characterized by a significantly higher endothelial and vascular area in comparison to adenoma and normal adrenal tissue, but no significant differences in vascular density between ACC and adenoma are observed [Sasano et al., 1998]. Therefore, Sasano et al. [1998] reasoned that ACC may show an increased proliferation of endothelial cells but no elevated intratumoral microvessels.

Mutation at the 17p13 locus harboring *TP53* is observed in approximately 25 % of ACC cases [Reincke et al., 1994]. Alterations of the 11p15 locus resulting in *IGF (insulin growth factor)-II* overexpression are frequently observed [Heppner et al., 1999]. In addition, Johnsen et al. [2009] showed that bone morphogenetic proteins 2 and 5 (BMP2, BMP5) are capable to inhibit proliferation and modulation of steroidogenesis *in vitro* which might regulate ACC biology since they are downregulated in this kind of cancer — in comparison to normal adrenal glands. BMPs are capable to induce endochondral bone formation [Hogan, 1996] but are also regulating cell growth, apoptosis and differentiation (reviewed in von Bubnoff and Cho [2001]). Additionally, they can act as autocrine or paracrine modifiers of tumor growth and function [Langenfeld et al., 2003]. Activating *CTNNB1* mutations are early and common events in benign and malignant adrenocortical tumors [Masi et al., 2009]. Kotoula et al. [2009] conclude that *BRAF, RAS* and *EGFR* genes are also mutated in a subset of adrenocortical carcinomas.

3.6.2 Clinical presentation of adrenocortical carcinoma

Approximately 60 % of ACC patients present with evidence of steroid hormone excess and Cushing syndrome is frequently observed. In the German ACC Registry 60 % of cases show autonomous cortisol secretion, either alone or in combination with other steroids [Koschker et al., 2006]. Average tumor size at presentation is about 12 cm and 35–40 % of these will be non-functional [Ng and Libertino, 2003, Kebebew et al., 2006].

The majority of patients presenting hormonally inactive adrenocortical carcinoma suffer from abdominal discomfort like vomiting and abdominal fullness or back pain resulting from large tumor mass burden. Moreover, an increasing percentage of ACC is discovered during abdominal imaging as incidentaloma [Barzon and Boscaro, 2000, Mantero et al., 2000].

3.6.3 Tumor staging of adrenocortical carcinoma

Since 2004 staging of ACC is performed according to the Union Internationale Contre le Cancer (UICC) published by the World Health Organization [DeLellis et al., 2004] which is based on MacFarlane [1958] and modified by Sullivan et al. [1978]. However, it was shown that the European Network for the Study of Adrenal Tumors (ENSAT) staging system is prognostically superior to the UICC staging system concerning ACC [Fassnacht et al., 2009, Lughezzani et al., 2010]. Table 3.1 shows the proposed ENSAT staging system.

Table 3.1: Proposed ENSAT tumor, nodes, and metastases (TNM) classification of adrenocortical carcinoma. Table according to Fassnacht et al. [2009].

Stage	TNM[a]
I	$T_1N_0M_0$
II	$T_2N_0M_0$
III	$T_{1,2}N_1M_0$ *or* $T_{3,4}N_{0,1}M_0$
IV	$T_{1,2,3,4}N_{0,1}M_1$

[a]For each stage defining features are given. T_1: tumor $\leqq 5$ cm. T_2: tumor > 5 cm. T_3: tumor infiltrates surrounding tissue. T_4: tumor invades adjacent organs or venous thrombus in vena cava or renal vein. N_0: no positive lymph node(s). N_1: positive lymph node(s). M_0: no distant metastases. M_1: distant metastases are present.

3.6.4 Histopathological diagnosis of ACC

Diagnostic pathology of adrenal tumors has to answer two main questions: *i.)* what is the origin of the tumor? and *ii.)* is the lesion benign or malignant? For the first question, the morphological distinction between ACC and metastatic carcinomas may be very demanding and it is strongly advised to involve a pathologist specialized in this field. Several molecular markers have been suggested but none reached general acceptance (see section 3.6.5). For the differential diagnosis benign *vs.* malignant adrenocortical tumors several multiparametric approaches have been proposed. Among these, the Weiss criteria are most widely utilized [Weiss, 1984, Weiss et al., 1989]. It differentiates benign and malignant tumors dependent on nine histolopathologic criteria. These criteria encompass, among others, nuclear grade, atypical mitosis, and invasion of neighbouring tissue. If three or more of these criteria are present, malignant behavior of the tumor is assumed [Weiss et al., 1989, Gicquel et al., 2001, Lucon et al., 2002]. Several other diagnostic criteria have been suggested for differentiation of benign and malignant adrenocortical tumors [Hough et al., 1979, van Slooten et al., 1985, Aubert et al., 2002, Bisceglia et al., 2004, Blanes and Diaz-Cano, 2007]. Furthermore, macroscopic evaluation of features like tumor weight, hemorrhaghe, and breached/intact tumor capsule are also used for differentiation.

3.6.5 Molecular markers

Immunohistochemistry is a powerful tool to differentiate between varying types of cancer. Therefore, it is not surprising that it was, and is, of special interest to evaluate and establish specific markers for discrimination of benign and malignant adrenocortical tumors as well as markers for differentiation of adrenocortical tumors from other retroperitoneal tumors.

Differentiation of malign and benign adrenocortical tumors can be achieved by Ki67 [Goldblum et al., 1993, Terzolo et al., 2001], which seems to be also of prognostic relevance since expression of > 10 % is associated with poorer survival (unpublished results from German ACC registry). Recently, Fenske et al. [2009] suggested glucose transporter GLUT1 and GLUT3 as useful marker for discrimination of adrenocortical adenoma and carcinoma — unfortunately, both are clearly below a positive staining percentage of 50 % in ACC. However, in contrast to Glut3, Glut1 is of prognostic value in malignant adrenocortical tumors [Fenske et al., 2009]. Epithelial cell adhesion molecule (EpCAM) does not seem to be expressed by ACC at all (group internal observations and unpublished results). The transcription factor SNAIL is expressed in

a subset of ACC and its expression is associated with decreased survival, advanced disease and higher risk for development of distant metastases [Waldmann et al., 2008]. Nakamura et al. [2009a] investigated the potential of vascular endothelial growth factor A (VEGFA), vascular endothelial growth factor receptor 2 (VEGFR2), epidermal growth factor receptor (EGFR), human EGFR-related 2 (HER2), ERK1/2, Akt, mammalian target of rapamycin (mTOR), p70S6 kinase, S6 ribosomal protein and 4E binding protein and concluded that *i.) EGFR* was significantly more abundant expressed in ACC than in benign ACT so that cetuximab (an EGFR antibody), gefitinib and erlotinib (both EGFR tyrosine kinase inhibitors) may provide clinical improvement, and, *ii.)* that VEGFA is associated with aggressive behavior in ACC. Last but not least, endocrine gland-derived vascular endothelial growth factor (EG-VEGF) and its receptors prokinecitin receptors 1 and 2 (PRK1, PRK2) are found in the vast majority of adrenocortical carcinomas (group internal observations and unpublished results).

For differentiation of adrenocortical tumors from other retroperitoneal tumors, some antigens were investigated. Recently, it was shown that, although membranously expressed by the vast majority of clear cell renal cell carcinoma, CD10 is not valuable for discriminating renal cell carcinoma from adrenocortical tumors [Mete et al., 2010]. Nestin, an intermediate filament protein, is not only expressed by a subset of adrenocortical tumors but also by neuroectodermal stem cells [Lachenmayer et al., 2009]. The well-proven ACT-specific marker D11 [Schroder et al., 1990, 1992, Tartour et al., 1993] is no longer commercially available (the only information available concerning the antigen of D11 is a not further specified human liver membrane protein of 59 kDa [Schroder et al., 1990]). Peptide hormone subunit inhibin-α (normally produced by ovarian granulosa and testicular Sertoli cells) was also shown to be specific for adrenocortical tumors [McCluggage et al., 1998, Munro et al., 1999] as well as melanoma antigen melan-A (MART1) [Busam et al., 1998, Ghorab et al., 2003]. A common characteristic of D11, inhibin-α, and melan-A is the observation of low sensitivity concerning corresponding antigens in adrenocortical carcinoma (see Table 3.2 on page 22). Sasano et al. [1995c, 2006] suggested SF1 as an useful marker for differentiation of adrenocortical and non-adrenocortical tumors. Almeida et al. [2010] published that *SF1* is overexpressed and more frequent in ACT from children than adults. Up to now, SF1's value as marker for ACC was only investigated in small series of 8, 5, and 4 samples, respectively [Sasano et al., 1995a,b, Kaneko et al., 2008]. Table 3.2 lists well-proven immunohistochemical markers used for diagnostical evaluation of adrenocortical tumors.

Table 3.2: Diagnostic markers D11, inhibin-α and melan-A for immunohistochemical differentiation of adrenocortical tumors from other adrenal masses as well as non-steroidogenic tissues. Shown are the antibody, references and entities used for immunohistochemistry. Furthermore, sensitivity, specificity, positive and negative predictive value estimated for adrenocortical carcinoma are shown, respectively. Regarding D11 nuclear staining was observed. In contrast, inhibin-α and melan-A (clone A103) showed cytoplasmic staining. Note the low sensitivity of each marker for its corresponding antigen in adrenocortical carcinoma. ACC: adrenocortical carcinoma. ACA: adrenocortical adenoma. NST: non-steroidogenic tumor.

	ACC		ACA		NST	
	+	-	+	-	+	-
D11 (5 references)[a]	76	28	135	0	1	274
For ACC sensitivity: 73 %, specificity: 99 %, positive predictive value: 98 %, negative predictive value: 90 %						
Inhibin-α (9 references)[b]	112	51	268	91	8	949
For ACC sensitivity: 69 %, specificity: 99 %, positive predictive value: 93 %, negative predictive value: 95 %						
Melan-A (6 references)[c]	100	38	233	17	11	1171
For ACC sensitivity: 72 %, specificity: 99 %, positive predictive value: 90 %, negative predictive value: 97 %						

[a] Komminoth et al. [1995], Schroder et al. [1990, 1992], Tartour et al. [1993], Wajchenberg et al. [2000]

[b] Arola et al. [2000], Fetsch et al. [1999], Jalali and Krishnamurthy [2005], McCluggage et al. [1998], Munro et al. [1999], Pan et al. [2005], Pelkey et al. [1998], Zhang et al. [2003, 2004]

[c] Busam et al. [1998], Ghorab et al. [2003], Jalali and Krishnamurthy [2005], Pan et al. [2005], Zhang et al. [2003, 2004]

3.6.6 Therapy of adrenocortical carcinoma

Unfortunately, adrenocortical carcinoma has not many options for treatment. Some of them are briefly introduced here.

Surgery Complete tumor removal (R0 resection) of stage I–III ACC is the treatment of choice, since it offers the best chance of cure [Icard et al., 1992, Lee et al., 1995, Dackiw et al., 2001]. Moreover, R0 resection seems to be associated with superior prognosis [Allolio and Fassnacht, 2006] which is supported by Grubbs et al. [2010] who also emphasized in a retrospective study that high-quality surgery also increases patients benefit — which, in turn, highlights that surgical resection of ACC should be performed by specialized surgeons at reference centers.

If adjacent organs are invaded by the tumor *en bloc* removal of these must be performed. Often, lymphadenectomy is also neccessary. Special attention must be paid to the fact that the tumor capsule must be left intact while operated, since breached capsules facillitate tumor spillage and local recurrences [Dackiw et al., 2001].

Surgery of local recurrences/distant metastases is further accepted and associated with improved survival in retrospective studies [Jensen et al., 1991, Schulick and Brennan, 1999a, Schteingart et al., 2005].

Radiotherapy and medical therapy Fassnacht et al. [2006] suggested postoperative irradiation of the tumor bed as valuable adjuvant therapeutic tool in ACC treatment. Furthermore, Allolio and Fassnacht [2006] recommended radiotherapy to control localized adrenocortical diseases not amenable to surgery.

Mitotane (o,p'DDD; commercially available as Lysodren; see Figure 3.6) is the only adrenal-specific chemical compound available for treatment of ACC. Asp et al. [2010] showed that each enantiomer is characterized by small but significant differences in its activity.

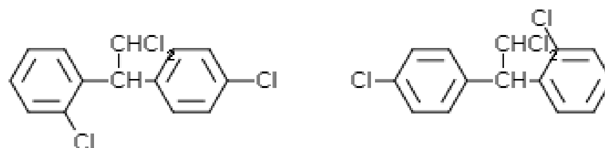

Figure 3.6: Enantiomers of mitotane.

After metabolic activation mitotane excerts its adrenolytic effect mainly as focal degeneration of *zona fasciculata* and *zona reticularis*. Few drug targets and actions are described [Tron'ko, 1970, van Seters and Moolenaar, 1991, Cai et al., 1997, Cabrini et al., 2001, Lindhe et al., 2002, Nader et al., 2006].

Currently, 5–6 g/day Lysodren are administered to patients suffering ACC at the University Hospital Würzburg depending on gastrointestinal tolerance [Allolio and Fassnacht, 2006]. After 14 days, dosage is controlled by drug plasma concentration and adjusted to 14 mg/ml. Berruti et al. [1992] and Terzolo et al. [2007] suggest that adjuvant mitotane might be the standard therapy in patients with ACC after complete tumor resection. In advanced ACC, objective response rates of mitotane seem to be of approximately 25 % of all cases [Hahner and Fassnacht, 2005]. Since mitotane is adrenolytic, it causes adrenal insufficiency and increases metabolic clearance of glucocorticoids [Hague et al., 1989], so that high-dose glucocorticoid substitution is needed.

Only a minority of patients seem to respond to cytotoxic chemotherapy protocols. Exceptions are treatment regimens combining mitotane with etoposide, doxorubicin and cisplatin [Berruti et al., 2005], less toxic regimens combining mitotane with streptozotocin [Khan et al., 2000], or vincristine, cisplatin, teniposide, and cyclophosphamide [Khan et al., 2004] as second-line chemotherapy after failure of streptozotocin plus mitotane. Recently, Ronchi et al. [2009] reported that patients expressing *excision repair cross complementing group 1 (ERCC1)* showed strong correlation with overall survival after platinum treatment.

Chacon et al. [2005] published that a certain patient who failed to respond to conventional mitotane-based therapy showed a partial response to the anti-angiogenic drug thalidomide. Another patient recieved, after failure of etoposide, doxorubicin, cisplatin and mitotane chemotherapy, the multi-targeted tyrosine kinase inhibitor sunitinib which lead first to a partial response followed by progressive disease [Lee et al., 2009]. Sperone et al. [2010] reasoned that gemcitabine (a nucleosid analog) plus metronomic 5-fluorouracil (capecitabine; an anti-metabolite) is a moderately active regimen in heavily pretreated ACC patients, since median time to disease progression was 5.3 months and overall survival 9.8 months, respectively.

3.7 Aims

Adrenocortical carcinoma is a rare malignancy characterized by general poor prognosis and heterogenous presentation [Wajchenberg et al., 2000, Dackiw et al., 2001]. Until now, no immunohistochemical marker showing high specificity for adrenocortical tumors, or their origin, is established and/or accepted yet. Thus, the questions we wanted to address with the present studies were the following:

1. Is SF1 useful as immunohistochemical marker for differentiation of adrenocortical and non-adrenocortical-originating retroperitoneal tumors, as suggested by Sasano et al. [1995c, 2006]? For this purpose, SF1 expression was investigated using tissue microarrays and full sections from 221 adrenal and 79 non-adrenal samples (including 163 ACC samples).

2. Is SF1 expression also of prognostic relevance in patients with ACC? To analyze this aspect, the immunohistochemical data were correlated with clinical data from the German ACC Registry.

3. Using fluorescence *in situ* hybridization, correlation between genomic variability and protein expression was analyzed. This aimed to prove if the known SF1 dosage-triggered effect on adrenocortical cell proliferation and cancer [Doghman et al., 2007] is rooted on a genetical basis or if other factors contribute to it — possibly enhanced stabilization of the SF1 protein, its protection of proteasomal degredation or by other proteins and mechanisms.

4. A possible SF1-mediated gene transcription of *FATE1* was immunohistochemically evaluated in adrenocortical and other retroperitoneal tumors, as well as the influence of the SF1 repressor DAX1, which genes harbour a putative SF1 binding site [Doghman et al., 2007, Burris et al., 1995].

—*Quidquid agis prudenter agas et respice finem.* [a]

[a] Whatever you do, do prudently [with forethought], and consider the goal.

4 Materials and methods

4.1 Equipment

Beecher Instruments, Silver Springs, USA
Tissue puncher/arrayer
Biometra, Göttingen, Germany
Gel documentation system BioDocAnalyze
Edmund Bühler, Hechingen, Germany
Incubator hood TH30, shaker SM 30
CardinalHealth, Ohio, USA
Flexam® sterile latex exam gloves
Daewoo Electronics Europe
Microwave oven Quick Cookmate
Eppendorf, Wesseling-Berzdorf, Germany
Research® pipets, 10 μl, 100 μl, 1000 μl, Safe-Lock tubes, 1.5 ml, 2 ml, BioPhotometer, UVette®
C. Gerhardt, Bonn, Germany
Magnetic stirrer
Greiner Bio-One, Frickenhausen, Germany
12 ml, 14 ml, 50 ml polypropylene tubes, Petri dishes
A. Hartenstein, Würzburg, Germany
Analytical balance Scaltec SBA 32, cover slips, table stopp watch, Parafilm® M, pH meter InoLab pH 720, tissue embedding cassettes, autoclaveable bags, inoculating loop
Heraeus, Hanau, Germany
Heating cabinet T5042E, table centrifuge Biofuge fresco
Jung, Heidelberg, Germany
Sliding microtome Hn40
R. Langenbrinck, Emmendingen, Germany
SuperFrost® plus silanized microscope slides
Leitz, Wetzlar, Germany
Aristoplan microscope
Liebherr, Biberach an der Riss, Germany
Fridge, 4 °C, − 20 °C
Medax Nagel, Kiel, Germany
Tissue flotation bath
Memmert, Schwabach, Germany
Incubator EB 400, water bath WB7

Noras, Würzburg, Germany
IKA® MS1 minishaker
Peqlab, Erlangen, Germany
Shaker Thriller
Sarstedt, Nümbrecht, Germany
Biosphere® filter tips, 10 μl, 100 μl, 1000 μl
SCI Science Services, München, Germany
Liquid blocker Super Pap pen
Schott, Mainz, Germany
Various glass ware
Scotsman, Vernon Hills, USA
Automatic ice machine AF-10
Stratagene, Amsterdam, Netherlands
Agarose gel electrophoresis chamber Joule Box™ System
Techne, Staffordshire ST15 OSA, UK
Heating block Dri-Block® DB 2D
Thermo Scientific, Langenselbold, Germany
Autoclave Varioklav®, NanoDrop 2000c
Weckert Labortechnik, Kitzingen, Germany
Moist chamber
Carl Zeiss AG, Oberkochen, Germany
Microscope Axiovert 135, signal calibrator FluoArc, camera AxioCam MRm, illumination unit HBO 100
ZVG, Troisdorf, Germany
zetBox® HighTechTissue

4.2 Chemicals, enzymes and sera

Unless otherwise stated all chemicals were purchased from Roth (Karlsruhe, Germany).
Dako, Glosdrup, Denmark
Liquid DAB+ substrate chromogen system
Invitrogen, Darmstadt, Germany
NotI
New England Biolabs, Frankfurt am Main, Germany
EcoRI
PAA, Colbe, Germany
Dulbecco's phosphate buffered saline
Roche, Mannheim, Germany
Blocking reagent for nucleic acid hybridization and detection, RNase
Sigma-Aldrich, Steinheim, Germany
Human AB serum, lot# 017K0443, phosphate buffered saline (PBS) tablets (0.137 M NaCl, 0.0027 M KH_2PO_4, 0.01 M phosphate buffer), pepsin
Vector, Burlingame, USA
NovaRED™ substrate kit, fluorescein-avidin-DCS

4.3 Deoxyribonucleic acid ladders

Fermentas, St. Leon-Rot, Germany
GeneRuler^{TM} 1 kb DNA Ladder showing fragment sizes (in bp) of 10000, 8000, 6000, 5000, 4000, 3500, 3000, 2500, 2000, 1500, 1000, 750, 500, 250
GeneRuler^{TM} 100 bp DNA Ladder showing fragment sizes (in bp) of 1000, 900, 800, 700, 600, 500, 400, 300, 200, 100
λ Mix Marker, 19 showing fragment sizes (in bp) of 48502, 38416, 33498, 29946, 24508, 23994, 19397, 17053, 15004, 12220, 10086, 8614, 8271

4.4 Antibodies and kits

Dako, Glosdrup, Denmark
Negative control N-Universal Negative Control Mouse, EnVision+® System Labeled Polymer-HRP Anti-Mouse, negative control N-Universal Negative Control Rabbit, EnVision+® System Labeled Polymer-HRP Anti-Rabbit
Machery-Nagel, Düren, Germany
NucleoSpin® Plasmid
Perseus Proteomics, Tokyo, Japan
Anti-human mouse monoclonal SF1 antibody, clone N1665, lot # A-2
Roche, Mannheim, Germany
Biotin-16-dUTP 5' dNTP kit
Santa Cruz Biotechnology, Heidelberg, Germany
Anti-human rabbit polyclonal DAX1 antibody, clone K-17, lot # E2108, anti-human mouse monoclonal FATE1 antibody, clone B5-9, lot # C1708

4.5 Preparation of equipment and solutions

Heat-stable equipment was (if neccessary) sterilized by autoclaving for 20 min. at 180 °C. Heat-stable solutions were sterilized by autoclaving for 20 min. at 121 °C. Heat-labile equipment were sterilized by cleaning using 70 % ethanol and subsequent drying. Heat-labile solutions were prepared as concentrated stock solutions and sterile filtrated through 0.2 μm membrane filters.

4.6 Buffers and solutions

Antibiotics were prepared and stored as published by Maniatis et al. [1984]. Tris-HCl buffers got pH adjusted by HCl whereas Tris-acetate buffers got pH adjusted by acetic acid.
Ampicillin
25 mg/ml (sodium salt) in *aqua destillata*. Sterile filtered, aliquoted and stored at − 20 °C.
Chloramphenicol
34 mg/ml in 100 % ethanol. Aliquoted and stored at − 20 °C.
Citric acid monohydrate
For 0.01 M citric acid monohydrate buffer 840 mg citric acid monohydrate were dissolved in 400 ml *aqua destillata* and adjusted to pH 6 using 2 N sodium hydroxide.

Eosin y
1 g eosin y was dissolved in 100 ml *aqua destillata*.
Ethidium bromide
Ethidium bromide stock solution (10 x) containing 10 mg/ml ethidium bromide.
4 % buffered formalin
6.5 g trisodium phosphate (0.04 M) and 4 g sodium dihydrogen phosphate monohydrate (0.033 M) were dissolved in 800 ml *aqua destillata*. 100 ml formalin (of a 40 % stock solution) were added and well mixed. Subsequently, pH was adjusted to pH 7.4 and poured in to 1000 ml with *aqua destillata*.
Hybridization mix for biotinylated FISH probes
1 g dextran sulfate (working concentration 10 %) was dissolved in 5 ml formamide and subsequently 5 ml 2 x SSC were added. Solvation was achieved on a stirrer at 50 °C. Aliquots were 1 ml each after sterile filtration using a syringe and 0.45 μm sterile filter. Aliquots were stored at − 20 °C. Solutions in use were stored at 4 °C.
Hydrogen chloride
0.01 M hydrogen chloride solution was prepared by mixing 990 ml *aqua destillata* and 10 ml 1 M hydrogen chloride.
Kanamycin
34 mg/ml in *aqua destillata*. Sterilization was achieved by filtration and aliquots stored at −20 °C.
6 x loading dye for nucleic acid agarose gel electrophoresis
60 % (w/v) sucrose, 0.1 % (w/v) bromphenol blue, 0.1 % (w/v) xylene cyanole FF were dissolved in 1 x TAE. By varying concentrations of bromphenol blue and/or xylene cyanole FF different colorings resulted.
Mayer's Hemalaun
Mayer's Hemalaun was prepared as published by Mayer [1891]. 1 g hematoxylin (0.003 M) was dissolved in 1000 ml *aqua destillata* under slight heating. Subsequently, 50 g aluminium-ammoniumsulfate dodecahydrate (0.11 M) and 0.2 g sodium iodate (0.001 M) were added and shaken until alum was completely dispensed. Hereafter, 1 g citric acid monohydrate (0.005 M) and 50 g chloral hydrate (0.302 M) were added and solubilized. The dilution had to mature at least 14 days.
PBS buffer
For immunohistochemistry phosphate buffered saline (1 x) was prepared by dissolving 5 PBS tablets in 1000 ml *aqua destillata*.

For fluorescence *in situ* hybridization a 10 x PBS solution was prepared by dissolving 80 g sodium chloride (1.37 M), 2 g potassium chloride (0.026 M), 16.02 g di-sodiumhydrogen-phosphate dihydrate (0.09M) and 2 g potassium dihydrogenphosphate in 1000 ml *aqua destillata*. pH 7.3 adjustment was performed using sodium hydroxide.
Pepsin solution
5 mg pepsin were dissolved in 100 ml 0.01 M hydrogen chloride.
RNase solution
10 mg RNase were solubilized in 100 ml 2 x saline sodium citrate (SSC).
SSC buffer
Saline sodium citrate buffer was prepared as 20 x stock solution. 175.32 g sodium chloride (3 M) and 88.2 g (1.08 M) sodium acetate were dissolved in 1000 ml *aqua destillata*. pH was adjusted to pH 7 using acetic acid. 2 x SSC solution was prepared by mixing 100 ml 20 x SSC and 900 ml *aqua destillata*.

Sodium acetate
2.5 M sodium acetate was dissolved in *aqua destillata* and subsequently adjusted to pH 5.2 using acetic acid.
Tetracycline
25 mg/ml were dissolved in ethanol/*aqua destillata* (50 % v/v). Aliquots were stored at − 20 °C.
TAE buffer
Tris-(hydroxymethyl)-aminomethane acetate ethylendiamine tetraacetic acid buffer was prepared as 50 x stock solution. 242 g Tris base (0.04 M), 100 ml 0.5 M EDTA (0.001 M) pH 6, 57.1 ml acetic acid (0.005 M sodium acetate) were mixed in 1000ml *aqua destillata*. If neccessary, pH was adjusted to pH 7.4.
Tris-EDTA buffer
Tris-(hydroxymethyl)-aminomethane ethylendiamine tetraacetic acid buffer (1 x) was prepared by dissolving 12.11 g (0.1 M) Tris Ultra and 1.49 g (0.004 M) EDTA in 2000 ml *aqua destillata*. Adjustment to pH 9 was performed by using sodium hydrochloride.
TN buffer
Tris-(hydroxymethyl)-aminomethane hydrogen chloride, sodium chloride buffer was prepared as 10 x stock solution. 121.4 g Tris (1.002 M) and 87.4 g sodium chloride (1.5 M) were dissolved in 1000 ml *aqua destillata*. Adjustment to pH 7.5 was performed by adding approximately 65 ml 37 % hydrogen chloride.
TNB buffer
1 x Tris-(hydroxymethyl)-aminomethane hydrogen chloride, sodium chloride, blocking solution buffer was prepared by dissolving 0.5 g blocking reagent in 10 ml 10 x TN and subsequently filled up to 100 ml using *aqua destillata*. 10 ml aliquots were stored at − 20 °C.
TNT buffer
1x Tris-(hydroxymethyl)-aminomethane hydrogen chloride, sodium chloride, Tween 20 buffer was prepared by mixing 50 ml 10 x TN buffer and 250 μl Tween 20 and subsequently filled up to 500 ml using *aqua destillata*.
Washing solution
95 ml *aqua destillata*, 2 ml 20 x SSC and 300 μl Tween 20 were mixed.

4.7 Patients

Adrenocortical tumor (ACT) tissue was collected from a cohort of 215 patients undergoing surgery of adrenocortical carcinoma (ACC, n=163) or benign tumors (n=52) such as aldosterone-producing Conn- (n=26), cortisol-producing Cushing- (n=16) or hormonally inactive- (n=10) adenomas. Diagnosis of ACC was based on established clinical, biochemical and morphological criteria [Allolio and Fassnacht, 2006] and all histological diagnoses, including Weiss score [Weiss et al., 1989] and Ki67 expression, were confirmed by the reference pathologist of the German ACC Registry (Wolfgang Saeger, Hamburg).

Clinical data of ACC patients including follow-up and survival data were collected in a structured manner by the German ACC Registry (`www.nebennierenkarzinom.de`) [Koschker et al., 2006]. For the present study, the follow-up information was locked as of January 2010. Tumor staging at the time of diagnosis was based on imaging studies, corroborated by the findings during surgery and reported according to the UICC/WHO classification 2004 [DeLellis et al., 2004]. The secreting status of neoplasms was investigated by suitable hormonal tests

[Fassnacht et al., 2004]. Surgical resection of the primary tumor was considered complete (R0 resection) if surgical, pathological and imaging reports did not show any evidence of remaining neoplasm-like tissues. Existence of distant metastases was evaluated at the time of diagnosis and during follow-up visits by computerized tomography of the chest and abdomen every 3–6 months. Patients gave informed consent for collecting tissue and clinical data, and the study was approved by the ethics committee of the University of Würzburg.

Non-steroidogenic tumor tissues (n=74) comprised carcinomas of renal (n=11), lung (n=12), breast (n=8), colon (n=7), pancreas (n=5), liver (n=7), prostate (n=4), endometrium (n=2), ovary (n=4), melanoma metastases (n=3), lymphomas (n=2), seminoma (n=1), and pheochromocytoma (n=8). Normal tissues (n=12) were normal adrenal glands (n=6) as well as normal ovary (n=6). Non-steroidogenic tumor as well as normal tissues were analyzed in an anonymous fashion in accordance with a general decision of the local ethics committee.

4.8 Immunohistochemistry

4.8.1 Introduction to immunohistochemistry

Immunohistochemistry (IHC) is a staining technique which permits specific staining of tissue parts by using enzymes which are linked to antibodies that recognize their epitopes directly or indirectly [Dalquen et al., 2000].

The process of fixation and embedding follows a certain protocol: after incubation in formalin, the fixative is washed out in water. Hereafter, tissues get dehydrated in alcohol (i.e. isopropanol) and placed in an intermediate medium (i.e. xylene). Finally, tissues get embedded in paraffin.

Positive and negative staining controls are very important and should always be used. The former ones are for proofing that staining procedures are performed correctly and antibodies are functional whereas the latter ones ensure that no unspecific reactions occur.

Antibodies (also known as immunoglobulins [Ig]) are grouped in the following subgroups: IgA, IgD, IgE, IgG and IgM which have different tasks in host defense (for an explanation of host defense see Abbas et al. [2007]). The main subtype used in IHC is IgG. Furthermore, IHC Igs are available as monoclonal and polyclonal proteins. The former is produced by one cell type (hybridoma, i.e. plasma tumor cell fused with one B cell) and recognizes only one special epitope of the used antigens whereas the latter is produced by different B cells of the host (thus, it is a mixture of natural antibodies) and recognizes different epitopes of the used antigens for antibody generation. Monoclonal antibodies are mainly produced in mice whereas hosts for generation of polyclonal Igs are, for example, rabbit, goat, pig, sheep, horse, and guinea pig.

After some washing steps, substrate for the enzyme is added whose catalyzation results in precipitation of color whereby antibody:antigen complexes are visualized [Welsch, 2006]. Samples that are part of the investigation have to be used as try-outs in order to determine the needed amount of antibody. This step prevents the samples from being over- or underdyed. In a last step tissues get counterstained and mounted in a medium which protects slices from environmental influences (i.e. light, bacteria, molds, and humidity).

Precipitation of color can be differentiated in membraneous, cytoplasmic and nuclear staining. Which kind is getting developed depends on the localization of the target protein. Occasionally, combinations are observable [Noll and Schaub-Kuhnen, 2000].

Semiquantitative scoring of immunostaining can be achieved by assessing average signal intensities on a certain scale, e.g. of 0–3 (0, negative; 1, weak; 2, medium and 3, highly positive) and the proportion of tumor cells showing a positive (nuclear) signal (0, 0 %; 0.1, 1–9 %; 0.5, 10–49 %; 1.0, $\geqq$ 50 %). Subsequently, intensity and proportion scores are multiplied to give an overall score [Al-Haddad et al., 1999, Handra-Luca et al., 2003].

Once a protocol is established it has to be followed strictly [Taylor et al., 1996].

4.8.2 Tissue microarrays and full sections

Tissue from a total of 156 formalin-fixed, paraffin-embedded adrenocortical carcinomas (125 primary tumors, 18 local recurrences and 13 metastases), 5 of each benigne Cushing (cortisol-producing), Conn (aldosterone-producing) and hormonally inactive adenomas, 1 phaeochromocytoma, 4 normal adrenal glands and two of each colon, ovarian, pancreas and prostata carcinoma was assembled into three tissue microarrays using a commercially available manual tissue puncher/arrayer according to the manufacturer's instructions.

Hematoxilin and eosin stained sections (see section 4.8.4) of formalin-fixed, paraffin-embedded tissue blocks were evaluated to identify representative areas of well-preserved morphology. The corresponding area on the block was marked for tissue punching. Five cores with a diameter of 0.6 mm were punched from each tissue block and arrayed into a recipient paraffin block at a distance of 1 mm between each core. Sections were cut and mounted (see section 4.8.3) on silanized slides also used for conventional immunohistochemical stains and dried for 30 min. at 56 °C.

We chose to array five punch biopsies per case to minimize the number of cases not analyzable due to tissue loss and to increase concordance rates among different cores. For each block, hematoxylin and eosin stained slides were cut to verify tumor cell content.

Additionally, full sections of paraffin-embedded tissue from 6 adrenocortical carcinomas (5 primary, 1 metastasis), 2 normal adrenal glands, 6 colon-, 2 endometrial-, 7 hepatocellular-, 11 non small cell lung-, 1 small cell lung-, 4 mamma-, 4 ovarian-, 3 pancreatic-, 2 prostate-, and 11 renal cell carcinomas together with 1 melanoma, 2 non-Hodgkin lymphoma and 1 seminoma as well as 6 normal ovaries and 1 palatine tonsil and 1 gastric mucosa were performed.

4.8.3 Cutting of formalin-fixed, paraffin-embedded tissue blocks and subsequent mounting on microscope slides

Formalin-fixed, paraffin-embedded tissue blocks were cut on a microtome to yield 1.5–4 μm thick tissue slices (full sections). Array slices were cut at 3 μm (conventional immunohistochemistry). For fluorescence *in situ* hybridization full sections were cut at 4 μm and array slices at 1 μm.

Tissue blocks were cooled down for at least 30 min. at − 20 °C (the advantage of cooling is that formalin-fixed, paraffin-embedded tissue blocks get smoother) and subsequently cut (if slices get wavy while cutting tissue blocks have to get cooled down again). Hereafter, slices were transferred into cold tap water; the darker surface faced up (the lighter side of the tissue slice adheres better on microscope slides). Here they were mounted on silanized microscope slides (while mounting they were simultaneously glazed using paint-brushes). Subsequently, mounted slices were slowly dipped (the slower the better) into a tissue flotation bath (40–50 °C) to receive the main glaze and were dried over night to get tightly bound to the microscope slides (this is important for antigen retrieval and prevention of floating off while washing

steps are performed). In the case of silanized microscope slides adherence is mediated by electrostatical forces.

4.8.4 Hematoxylin-eosin staining

Hematoxylin-eosin staining was performed as follows: slices were deparaffinized twice in 100 % xylene for 10 min. and rehydrated by subsequent incubation for 10 min. each in 100 %, 90 %, 80 % and 70 % ethanol, followed by an extensive washing step with *aqua destillata*. Tissue sections were stained for 5–10 min. in Mayer‘s Hemalaun at RT. Nuclei blueing was performed in running tap water for 5 min. and subsequently rinsed with *aqua destillata*. Eosin solution was diluted 1:10 with *aqua destillata* and an incubation step of 5–10 min. performed at RT (in case that the dye will not infiltrate the tissue sections add 1 drop glacial acetic acid to 100 ml of eosin dilution). Sections were rinsed with *aqua destillata* and subsequently dehydrated for maximally 10 s in 100 % ethanol (EtOH). Hereafter, a 20 min. drying step was performed at 56 °C. Tissue slices were mounted in Entellan® and sealed with cover slips.

This method is mainly used to identify representative areas of given tumors as well as to differentiate them from normal tissue. For good representation it is important to choose tumor areas comprised of mainly tumor tissue and as few connective tissue as possible.

4.8.5 SF1 protein staining

TMAs and full sections were deparaffinized twice in 100 % xylene for 10 min. Rehydration was achieved by subsequently incubating tissues for 10 min. in 100 %, 90 %, 80 % and 70 % EtOH each, followed by an extensive washing step with *aqua destillata* at room temperature (RT). Antigen retrieval was performed by boiling slides in 10 mM citric acid monohydrate buffer pH 6 twice for 5 min. at 750 watt (W) in a microwave oven. After the buffer was cooled down (20 min.) TMAs or full sections were washed five times with *aqua destillata*. To minimize application of antibodies and solutions, tissue slices were encircled using a liquid blocker (pap pen). Quenching of endogenic peroxidase was achieved by an incubation step of 10 min. in 3 % hydrogen peroxide solution in methanol at RT followed by extensive washing with *aqua destillata*. Subsequently, blocking of unspecific protein:antibody interactions was performed using 20 % human AB-Serum in Dulbecco‘s phosphate buffered saline (DPBS) for 1 hour at RT. SF1 protein was detected by monoclonal mouse anti-human SF-1 antibody diluted 1:100 (in 3 full sections 1:50) in DPBS. Incubation of the primary antibody and corresponding negative control N-Universal Negative Control Mouse was performed over night in a humidity chamber at 4 °C. After incubation, tissues were washed five times with DPBS. Signal amplification was achieved by EnVision+® System Labeled Polymer-HRP Anti-Mouse for 30 min. at RT and developed for 7 min. at RT with NovaRED™ substrate kit according to manufacturer's instructions. After rinsing the slides with *aqua destillata*, nuclei were counterstained by Mayer's Hemalaun for 2 min. at RT. Blueing of nuclei was achieved in running tap water for 5 min. Subsequently, slices were dehydrated in 100 % EtOH and dried for 20 min. at 56 °C. Hereafter, they were mounted in Entellan® and sealed with cover slips. Only nuclear protein staining was considered 'positive staining' [Sasano et al., 1995b,c].

4.8.6 FATE1 protein staining

Handling of tissue slices is performed as described in the SF1 staining procedure (section 4.8.5). Differences were that FATE1 protein was detected by monoclonal mouse anti-human

fetal and adult testis expressed 1 antibody diluted 1:50; signal development was achieved for 10 min. with DAB according to manufacturer's instructions at RT. Only cytoplasmic protein staining was considered 'positive staining' [Yang et al., 2005].

4.8.7 DAX1 protein staining

Handling of tissue slices was performed as described in section 4.8.5. Differences were that DAX1 protein was detected by polyclonal rabbit anti-human DAX1 antibody diluted 1:1000 (performing multiple dilution steps) and that N-Universal Negative Control Rabbit was used as negative control. Only nuclear protein staining was considered 'positive staining' [Sato et al., 2003, Nakamura et al., 2009b].

4.8.8 Microscopical analysis of IHC tissue slices

TMAs and full sections were analyzed independently by two investigators with an Aristoplan microscope and the different reaction intensities were given values from 0 (stain-negative) to 1 (low), 2 (moderate) and 3 (highly positive), unless otherwise indicated. Where discrepancies were observed, results were double-checked by a third observer.

A semiquantitative H-score [Olaussen et al., 2006, Ronchi et al., 2009] was used, the percentage of positive cells of each specimen was estimated and subsequently, a proportional score allocated (0 for 0 % positivity, 0.1 for 1–9 % positivity, 0.5 for 10–49 % positivity and 1 if $\geqq$ 50 % were positive) and afterwards, this proportional score was multiplied by the staining intensity.

4.9 Fluorescence *in situ* hybridization

4.9.1 Introduction to (fluorescence) *in situ* hybridization (FISH)

The first *in situ* hybridization techniques relied on autoradiographical detection of abundant nucleic acid sequences like polytene chromosomes or highly reiterated sequences on metaphase chromosomes [Gall and Pardue, 1969, John et al., 1969, Evans et al., 1974]. To surpass limitations associated with autoradiography, e.g. time consumption, special handling of radiographic materials, several nonradioisotopic alternatives were developed (e.g., biotin-avidin beads, direct labeling of fluorochromes to DNA and biotynilated dUTP into DNA probes) [Rudkin and Stollar, 1977, Broker et al., 1978, Baumann et al., 1980, 1981, Langer et al., 1981]. Kislauskis et al. [1993] reported that labeling of synthetic, single-stranded DNA probes carrying enough fluorescent molecules for direct detection is possible. Furthermore, Henegariu et al. [2001] suggested that chemical aging is a rapid slice pretreatment sufficient for applying freshly prepared cytogenetic slides to *in situ* hybridization within 30 min. and that gradually denaturing of fresh biologic tissue allows its use for FISH while chromosomal integrity is protected.

One may divide *in situ* hybridization into three main parts, *i.)* preservation of the target sequence in an accessible state which is critical for ribonucleic acids, *ii.)* high efficiency hybridization of probe and target molecules but without significant unspecific adherence which would result in background staining and false-positives, *iii.)* specific probe detection to give detectable signals with minimal background staining (see Figure 4.1).

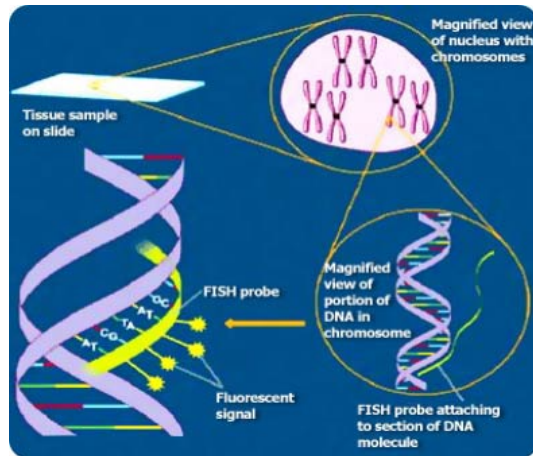

Figure 4.1: Schematical overview of FISH. A fluorescent-labeled nucleic acid probe attaches to a complemetary strand of chromosomal double-stranded DNA. Furthermore, it is indicated that probe and target sequence form Watson-Crick base pairs which results in specific fluorescence-mediated signals. Figure taken from `http://www.ivfsurrogacy.com` in June, 2010.

4.9.2 Probe preparation for fluorescence *in situ* hybridization

Culture of *Escherichia coli* For establishing and maintenance of *Escherichia coli* (*E. coli*) cultures the following steps were performed: Lysogeny broth (LB) media (0.5 % (w/v) yeast extract, 1 % tryptone, 1 % sodium chloride) [Bertani, 1951] were autoclaved for 20 min. at 121 °C. For solid media 1.5 % agar were added. Additives (e.g. antibiotics) were added after cooling down to approximately 40 °C. Agar plates were stored overhead at 4 °C. Before usage they were brought to room temperature.

Antibiotics were added from a 1000fold concentrated stock solution to a final concentration of 100 μg/ml ampicillin, 75 μg/ml kanamycin, 25 μg/ml tetracyclin. Chloramphenicol had an end concentration of 12.5 μg/ml (concentration of stock solution: 34 mg/ml).

Culture and storing of *Escherichia coli* To each culture medium an antibiotic was added to which transformed *E. coli* was *plasmidic*- and not genomic-mediated resistant. This ensures that the vector is not degraded by the host cell. Strains were grown over night at 37 °C. Volumina up to 5 ml were handled in culture vials and up to 50 ml in Erlenmeyer flasks on a shaker under an incubator hood. For short term storage (a few days to a couple of weeks) strains were streaked out on agar plates with the appropriate antibiotic. After incubation over night at 37 °C plates were stored at 4 °C.

Long term storage was achieved by mixing liquid bacteria culture 1:1 with 50 % glycerol in a cryo vial. After vortexing the mixture quickly, these stocks were stored at − 80 °C. From liquid cultures new stocks were obtained by streaking out the bacteria on agar plates to ensure the permanent availability of viable *E. coli*.

Genotype of *Escherichia coli* DH10B bacpac clone RP11-91G7 For SF1 fluorescence *in situ* hybridization we purchased *Escherichia coli* strain DH10B cloned with plasmid pBACe3.6. This vector carries a human chromosomal section comprising 9q33–9q34. As selection marker this strain is vector-mediated resistant to chloramphenicol and was grown in LB medium containing 12.5 μg/ml chloramphenicol.

The plasmid pBACe3.6 is a bacterial artificial chromosome (BAC) vector initially engineered by Shizuya et al. [1992] and by Children's Hospital Oakland Research Institute (CHORI) improved. Amongst others, the *sacBII* gene from *Bacillus amyloliquefaciens* (derived from Nat Sternberg's P1 vector [Pierce et al., 1992]) was cloned into it to serve as a positive-selection marker; furthermore, pBACe3.6 is a high copy number vector [Frengen et al., 1999]. Plasmid composition is shown in Figure 4.2.

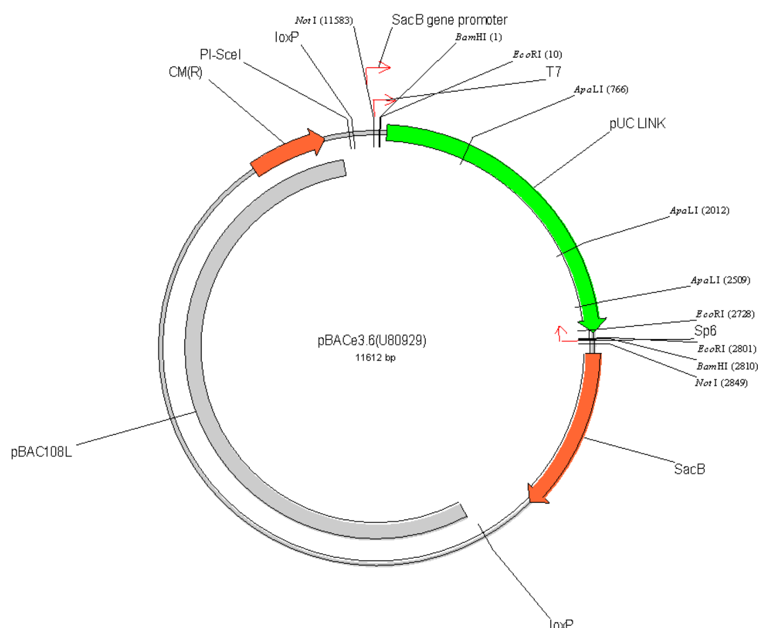

Figure 4.2: Graphical representation of vector pBACe3.6, gene cassette *sacBII*, stuffer fragment pUC LINK and pBAC108L plasmid-part. Furthermore, recognition sites for various restriction endonucleases are shown. Figure taken from `http://bacpac.chori.org` in June, 2010.

The genotype of *E. coli* strain DH10B is: F$^-$ endA1 recA1 galE15 galK16 nupG rpsL ΔlacX74 ϕ80lacZΔM15 araD139 Δ(ara,leu)7697 mcrA Δ(mrr-hsdRMS-mcrBC) λ^-. This means that the strain DH10B does not carry the F plasmid (F$^-$), endonuclease 1 is elimimated (endA1), it is deficient in DNA repair, increased competence due to a mutation at position 15 of galE (galE15), can not metabolize galactose and is resistant to 2-deoxygalactose due to a mutation (galK16). Furthermore, it is capable of uptaking large plasmids (nupG), is mutation-mediated streptomycin resistant (rpsL), and the entire *lac* operon as well as some flanking region is deleted (ΔlacX74). Moreover, it carries a defective version of phage ϕ80 carrying lacZM15 deletion, the arabinose metabolism is blocked due to mutation at position 139 (araD139), carries chromosomal deletions of the genes responsible for arabinose and leucine (Δ(ara,leu)7697), and is unable of restrictioning methylated DNA at sequence C^{m}CGG due to mutation (mcrA). Furthermore, this strain shows chromosomal deletions of the genes responsible for restriction of methylated DNA at the sequence C^{m}AG or G^{m}AC, methylation of certain sequences is deleted, and for efficient transformation of cloned unmethylated DNA from PCR amplifications and eliminated restriction of methylated DNA at the sequence R^{m}C (Δ(mrr-hsdRMS-mcrBC)), and is lambda negative (λ^-).

Plasmid isolation of *Escherichia coli* in analytical scale Isolation of plasmid pBACe3.6 in analytical scale (mini prep) was performed using NucleoSpin® plasmid kit (Machery-Nagel, Düren, Germany) according to manufacturer's instructions. In principle, the protocol follows the alcalic sodiumdodecyl sulfate lysis [Birnboim and Doly, 1979].

Inoculation of 3–5 ml nutrient solution druged with appropiate antibiotic with *E. coli* cells from a cryo culture or agar plate was performed. After an incubation over night at 37 °C 1 ml of saturated *E. coli* culture was transferred into a fresh Eppendorf tube and pelleted for 30 s at 11000 x g at RT. As much supernatant as possible was removed. Cell lysis was achieved by applying 250 μl A1 buffer and subsequent resuspension of the pellet. Afterwards, 250 μl A2 buffer was added and gently mixed by inverting the tubes for 6–8 times, followed by an up to 5 min. incubation step (lysate should appear clear). Subsequently, 300 μl A3 buffer was added and mixed gently by inverting the tubes for 6–8 times. Lysate was cleared (i.e. cell debris removal) by centrifuging for 5 min. at 11000 x g at RT. This step was repeated if no clearance was observed. Binding of deoxyribonucleic acid (DNA) to the silica membrane was performed by decanting the supernatant onto the column and subsequent centrifugation for 1 min. at 11000 x g at RT. Flow-through (FT) was discarded. An additional washing step with 500 μl AW buffer preheated to 50 °C was performed. FT was discarded after centrifuging for 1 min. at 11000 x g at RT. The membrane was washed by applying 600 μl A4 buffer onto the column, centrifuged at 11000 x g at RT, and the FT discarded. Subsequently, the silica matrix was dried by centrifuging 2 min. at 11000 x g at RT. DNA elution was performed by pipetting 50 μl elution buffer AE (preheated to 70 °C) onto the column and subsequent incubation for 2 min. at 70 °C followed by a centrifugation step for 1 min. at 11000 x g at RT. Mean recovery of plasmid-nucleic acid was around 35 ng/μl. Optical density (OD) ratios for each plasmid isolation were approximately $OD_{260/280} = 1.68$ and $OD_{260/230} = 1.7$.

Precipitation of deoxyribonucleic acid from aquous solutions Precipitation of DNA was achieved by using 1/10 vol. 2.5 M sodium acetate pH 5.2 and 3 vol. ice-cold ethanol (96 %). Subsequently, precipitation was performed over night at − 20 °C. DNA was pelleted by centrifugation (16060 x g, 30 min., 4 °C). Supernatant was discarded and the pellet washed with 70 % ethanol to remove remaining salts. After centrifugation (16060 x g, 15 min., 4 °C) the supernatant was discarded and the pellet dried for approximately 20 min. at 37 °C. The DNA pellet was resuspended in an appropriate volume of *aqua destillata* or TE buffer. Storage of DNA was done at 4 °C or − 20 °C for short term, or − 80 °C for long term periods.

Analytical agarose gel electrophoresis Size-based separation of DNA was achieved by applying a field force of 5–10 volt per centimeter (V/cm) which equals 60–120 V (1 % agarose gels). 0.7 % agarose gel electrophoresis was performed by applying 15 V over night. Staining of nucleic acid was performed by 10 μg/ml ethidium bromide. Afterwards, visualization and photography was accomplished by a gel documentation system for visual inspection.

Determination of nucleic acid concentration DNA concentration was measured photometrically using NanoDrop 2000c (Thermo Scientific, Langenselbold, Germany) according to manufacturer's instructions. Considering the ratio OD_{260}/OD_{280}, one can estimate the degree of DNA purity since deoxyribonucleic acids absorp light at 260 nm and proteins at 280 nm. *Aqua destillata* or buffer acted as reference.

Digestion of deoxyribonucleic acid by restriction endonucleases Plasmids were digested using 5–10 U (one U specifies enzyme activity which turns under specified conditions 1 μmol substrate per minute and milligram protein over) per microgram DNA for 2 hours at recommended temperature. If restriction assays were performed over night, less enzyme was used. Heat-labile enzymes got inactivated at appropriate temperature. Quality of performed assays was verified by analytical gel electrophoresis.

Preparation of biotynilated 16-dUTP probes for fluorescence *in situ* hybridization The nick translation method is based upon the fact that low concentrations of DNase I hydrolyses one DNA strand ('nicks' are formed) under low magnesium chloride conditions [Rigby et al., 1977]. These nicks are used by *E. coli* DNA-polymerase I to synthesize new complementary strands using the nicked ones as template (DNA-polymerases always needs a free 3'-OH end) [Kelly et al., 1970]. DNA-polymerase I's proof-reading capability (5'→3' exonuclease activity) removes nucleosid phosphates in direction of the nascent strand [Klett et al., 1968] which are substituted by hapten-labeled nucleotides [Rigby et al., 1977].

Biotin-16-dUTP nick translation probe preparation for fluorescence *in situ* hybridization was achieved using Biotin-16-dUTP 5' dNTP kit (Roche, Mannheim, Germany) following manufacturer's instructions. 1 μg template DNA was brought to a final volume of 16 μ with *aqua destillata*. 4 μl Biotin-Nick Translation mix were added, merged, and shortly centrifuged. Hereafter, 90 min. incubation at 15 °C was performed and the reaction stopped by adding 1 μl EDTA and subsequent incubation for 10 min. at 65 °C. Verification of obtained fragment size (expected were around 200–500 nucleotides) was achieved by agarose gel electrophoresis. For this purpose, 3 μl of each reaction assay was mixed with loading dye and incubated for 3 min. at 95 °C, followed by a 3 min. cooling down step on ice and subsequent gel electrophoresis.

4.9.3 *SF1* fluorescence *in situ* hybridization

Tissues used for fluorescence *in situ* hybridization comprised 5 normal adrenal glands, 2 aldosterone-producing, 2 cortisol-producing, 1 endocrine-inactive adenoma, 1 hyperplasia, 20 primary adrenocortical carcinomas, 1 local recurrence with matched primary malignant tumor, and 2 metastases with matched primary carcinoma.

Hotplate and pepsin solution were pre-heated to 80 °C and 37 °C, respectively. Formalin-fixed, paraffin-embedded tissue slides (max. 10) were incubated for one hour at 80 °C to liquify paraffin. Slides were then incubated twice in 100 % xylene at RT for 10 min., followed by rehydration for 5 min. with each 100 %, 90 % and 70 % EtOH at RT. Tissues were washed for 5 min. in 1 x PBS and subsequently boiled in a pressure cooker for 5 min., followed by a 20 min. cooling down step. Tissues were washed for 5 min. in 1 x PBS. Subsequently, RNase digestion for one hour at 37 °C and incubation for 5 min. at RT in 2 x SSC was performed. Nuclear membrane digestion was achieved using pre-heated pepsin-solution for 70 min. at 37 °C. Enzyme activity was abolished by incubating tissues 5 min. in 1 x PBS at RT. Tissue dehydration was performed by incubating slices in 70 %, 90 % and 100 % EtOH for each 5 min., followed by a drying step for 15 min. at 37 °C. For denaturing deoxyribonucleic acid slides were incubated 10 min. at 80 °C *after* applying a total volume of 10 μl comprised of probe solution and hybridization mix buffer (4 or 6 μl probe plus 6 or 4 μl hybridization mix buffer, respectively; depending on probe concentration) onto slices and subsequent sealing using Fixogum. Incubation was performed over night in a moist chamber at 37 °C. The next day, water bath and washing solution were pre-heated to 73 °C. Fixogum was removed

carefully and two washing steps were performed in 2 x SSC followed by 3 x 3 min. incubation in washing solution at 73 °C avoiding over-incubation as it might also remove probes. Blocking was performed with 200 μl TNB buffer per slide for 45 min. in an air tight humidity chamber at 37 °C. Subsequently, 2 μl fluorescein-avidin-DCS in 200 μl TNB buffer was added, slides sealed with Parafilm and an incubation for 30 min. in air tight moist chamber at 37 °C performed. Afterwards, slides were washed twice with 1 x TNT buffer 5 min. at RT. Tissue dehydration was achieved by 4 min. incubation in 70 %, 90 %, and 100 % EtOH each at RT. Slides were dried for 10–15 min at 37 °C (alternatively 20 min. at RT). Subsequently, 1 drop Vectashield with DAPI was added onto tissues and sealed with cover slips. Slides were stored in the dark at 4 °C.

4.9.4 Microscopical analysis of FISH

Evaluation of *SF1* gene copy number was performed according to Pianovski et al. [2006]. For each specimen, at least two distinct areas were photographed and subsequently evaluated. Estimation of gene copy number was performed by counting at least 50 nuclei. Subsequently, nuclei were counted presenting 1, 2, 3, 4, and > 4 specific fluorescent signals, respectively. Tissues were considered as normal diploid for *SF1* if $\geqq 50$ % of cell nuclei were characterized by 2 FISH signals and decreased gene copy number was considered positive if $\geqq 50$ % of cell nuclei were positive for 1 FISH signal. Increased gene copy number was considered as true if $\geqq 30$ % of nuclei presented 3 specific fluorescent signals and amplification of *SF1* gene was assumed if $\geqq 30$ % of nuclei presented $\geqq 4$ specific fluorescent signals.

4.10 Statistical evaluation of data

Overall survival was defined as patient lifespan after surgical resection of the primary tumor. European Network for the Study of Adrenal Tumors (ENSAT) I–III recurrence-free survival was defined as the time course from complete surgical resection (R0 resection) of the primary tumor to recurrence of tumor. Patients still alive or without recurrence, respectively, were censored. The statistical analysis was performed using SPSS software. All tests were two-tailed and a P value less than 0.05 was considered as statistically significant for each analysis.

4.10.1 Mantel-Cox test

Statistical analysis of patient survival was performed by the Mantel-Cox test (logrank test) [Mantel, 1966, Peto and Peto, 1972]. For the multivariate analysis, age, ENSAT stage, and sex were included as covariables. Hormonal status was ignored — in contrast to our published results [Sbiera et al., 2010] — to ensure sufficient sample sizes. Survival analysis was only performed for ACC patients presenting primary tumors for whom sufficient follow-up data were available (n=130).

The logrank test is a hypothesis test which compares survival distributions of two samples. It is a non-parametric test and appropriate to use if data are rightly censored. It compares hazard functions (also known as failure rate, hazard rate, or force of mortality) of two groups at each observed time point. It computes the observed and expected number of events in one of the groups at each observed event time. Subsequently, these are added to obtain an overall summary across all time points where there is an event. The logrank test compares

each observed number of events in the groups, respectively, at given time to its expectation under the null hypothesis.

4.10.2 Kaplan-Meier test

The Kaplan-Meier estimation [Kaplan and Meier, 1958] is based on the assumption (actually it is used for estimating the probability) that within a given time interval a certain event does not happen. It indicates patients at which the event at the given time point occurs. The confidence interval (CI, also known as Greenwood's formula [Greenwood, 1926]) puts a standard error on the Kaplan-Meier estimator using the delta-method. Kaplan-Meier survival analysis was carried out for calculating median duration (and 95 % CI) of overall as well recurrence-free survival. Patients were censored if they were alive or disease-free at the time of their last visit.

4.10.3 Cohen's κ and Pearson's ρ

For estimating the inter-rater agreement Cohen's κ [Cohen, 1960] was used. The numerator is the difference of observed and coincidentally observed agreement. The denominator is for standardization/normalization. The maximal value of κ is 1 (total agreement). $\kappa = 0$ corresponds to only coincidental agreements. Since negative values are also possible and indicate agreement below coincidence level, they should be treated as coincidences as well [Brennan and Silman, 1992].

Pearson's ρ [Pearson, 1896] (also known as correlation coefficient, bivariate correlation coefficient and zero-order correlation coefficient) for estimating the linear relationship of both observers was used for estimating the quality of match of the classifications of both observers. Pearson's ρ ranges from -1 to +1. The former indicates a perfect negative and the latter a perfect positive correlation. If $\rho = 0$, both sequences (of observations) are completely independant.

A strong agreement cutoff of 0.81 for Cohen's κ and 0.75 for Pearson's ρ was choosen [Landis and Koch, 1977, Coenraads et al., 2005].

—*One is a novelty. Two is a coincidence. Three is a problem.*
Martin Jeffrey, pathologist, Central Veterinary Laboratory, co-discoverer of BSE

5 Results

5.1 Overall patients characteristics

Adrenocortical adenomas were grouped in aldosterone- (APA; n=26), cortisol- (CPA; n=16), and endocrine-inactive (EPA; n=10) adenomas. Mean age of APA, CPA, and EPA patients was 51, 49, and 65 years, respectively. Mean tumor size was 1.9, 3.4, and 3.9 cm, respectively, see Table 5.1.

Within the adrenocortical carcinoma (ACC) group 133 samples were primary tumors, 19 local recurrences, and 15 distant metastases. For primary adrenocortical carcinoma mean age was 50 years. ENSAT stages I–IV were characterized by mean ages of 62, 48, 53, and 47 years, respectively. Mean age of patients presenting local recurrences and distant metastases was 50 and 45 years, respectively. Concerning primary adrenocortical carcinoma mean tumor size was 12 cm. Mean tumor size of ENSAT stages I–IV was 5, 12, 12, and 13 cm, respectively.

Table 5.1: Patients and tumor characteristics of adrenocortical tissues. Age and size are mean values. SD: standard deviation. Table according to Sbiera et al. [2010].

Tissue	Age [yr (SD)]	Sex (m/f)	Size [cm (SD)]
Normal adrenal gland (n=6)	59 (16)	4/2	-
Adrenocortical adenoma			
Aldosterone-producing ACA (n=26)	51 (11)	17/9	1.9 (1)
Cortisol-producing ACA (n=16)	49 (12)	4/12	3.3 (1)
Endocrine-inactive ACA (n=10)	65 (10)	6/4	3.9 (3)
Adrenocortical carcinoma[a]			
Primary (n=133[b])	50 (16)	48/85	12 (4)
Stage I (n=5)	62 (24)	2/3	5 (0)
Stage II (n=48)	48 (17)	19/29	12 (5)
Stage III (n=40)	53 (14)	14/27	12 (4)
Stage IV (n=34)	47 (18)	11/23	13 (4)
Local recurrences (n=19)	50 (18)	9/10	-
Distant metastases (n=15)	45 (12)	3/12	-

[a] Staging was performed as suggested by ENSAT classification (Fassnacht et al. [2009]).

[b] In 6 cases tumor stage was not determined, 48 ACC were cortisol-secreting, 13 purely sex-hormone or precursor secreting, 7 aldosterone-secreting and 18 hormonally inactive. In 48 cases no sufficient endocrine work up was performed preoperatively or data was not available.

For diagnostic discrimination of non-steroidogenic tumor tissues which metastasize to adrenal glands (e.g. melanoma, breast, gastrointestinal, lung, and renal cancer [Abrams et al., 1950, Lloyd et al., 2004]) from ACC, and tissues invaded by adrenocortical carcinoma (e.g. liver) such tissues were chosen for evaluation (see below). Pheochromocytoma was investigated since it is sometimes difficult to histologically discriminate it from ACC.

Since non-steroidogenic tumor and normal tissues were anonymized in accordance with the local ethics committee (see section 4.7 on page 29) no detailed information about patient characteristics was available. However, tissues were comprised of carcinomas of kidney (n=11), lung (n=12), breast (n=8), colon (n=7), pancreas (n=5), liver (n=7), prostate (n=4), endometrium (n=2), ovary (n=4), melanoma metastases (n=3), lymphomas (n=2), seminoma (n=1), and pheochromocytoma (n=8). Normal tissues were comprised of normal adrenal gland (n=6) and ovary (n=6).

5.2 Immunohistochemical staining of SF1, FATE1, DAX1 and their diagnostic value

5.2.1 SF1 protein staining

Evaluation of immunohistochemical stained tissues revealed prominent nuclear localization of SF1. Furthermore, homogeneous distribution of SF1 protein was observed. Both normal adrenal gland and ovary (each n=6) specimens showed protein expression (as expected) in each sample. All three cortical zones were positive — capsule and medulla negative (Fig. 5.1 A1–A3 on page 42). Within the adrenal cortex strong staining was present in *zonae glomerulosa & fasciculata* whereas *zona reticularis* was low (Fig. 5.1 A2 on page 42). Contrary to steroidogenic active theca and granulosa cells of normal ovary with strong staining, other cell types of this organ were negative (Fig. 5.1 F on page 42).

SF1 protein expression was found in all investigated benign neoplasms of adrenocortical origin (Figure 5.1 C page 42 and Table 5.2 on page 43). In addition, 158 of 161 (98 %) of ACC were positive for SF1, from which 49 samples (30 %) revealed high staining intensity (Fig. 5.1 D on page 42). From these, 43 were primary ACC samples, 4 local recurrences and 2 distant metastases. ACC tissues presenting low SF1 positivity (n=109; 68 %) were 84 primary ACC, 14 local recurrences and 11 distant metastases. 2 % of ACC samples (three primary tumors) showed no SF1-specific immunoreactivity. For statistical reasons the 3 negative adrenocortical carcinoma samples were included in the low SF1 expression group.

All 73 non-steroidogenic examined tissues were SF1 negative. Notably, in seminoma only steroidogenic active Leydig cells showed strong immunohistochemical staining whereas tumor tissue was negative for SF1 (Fig. 5.1 L on page 42). In addition, as shown in Figure 5.1 M on page 42, renal clear cell carcinoma tissue was negative for SF1 protein staining — in contrast to adjacent SF1 positive adrenocortical cells.

Table 5.2 on page 43 shows a summary of all tissues in which SF1 protein expression was investigated by IHC.

Furthermore, SF1 staining intensity was independent of endocrine activity as well as tissue (primary tumor, local recurrence, distant metastases; Table 5.3 on page 43).

Interobserver agreement (Cohen' s $\kappa = 0.92$; 95 % CI = 0.87–0.97) as well as linear relationship of both observers (Pearson's $\rho = 0.86$; 95 % CI = 0.82–0.89) was strong (see section 4.10.3 for explanation).

Considering all tumor tissues derived from non-steroidogenic organ entities and ACC samples for analysis of SF1 expression, sensitivity, specificity, positive predictive value, and negative predictive value were 98.6 %, 100 %, 100 %, and 97.3 %, respectively.

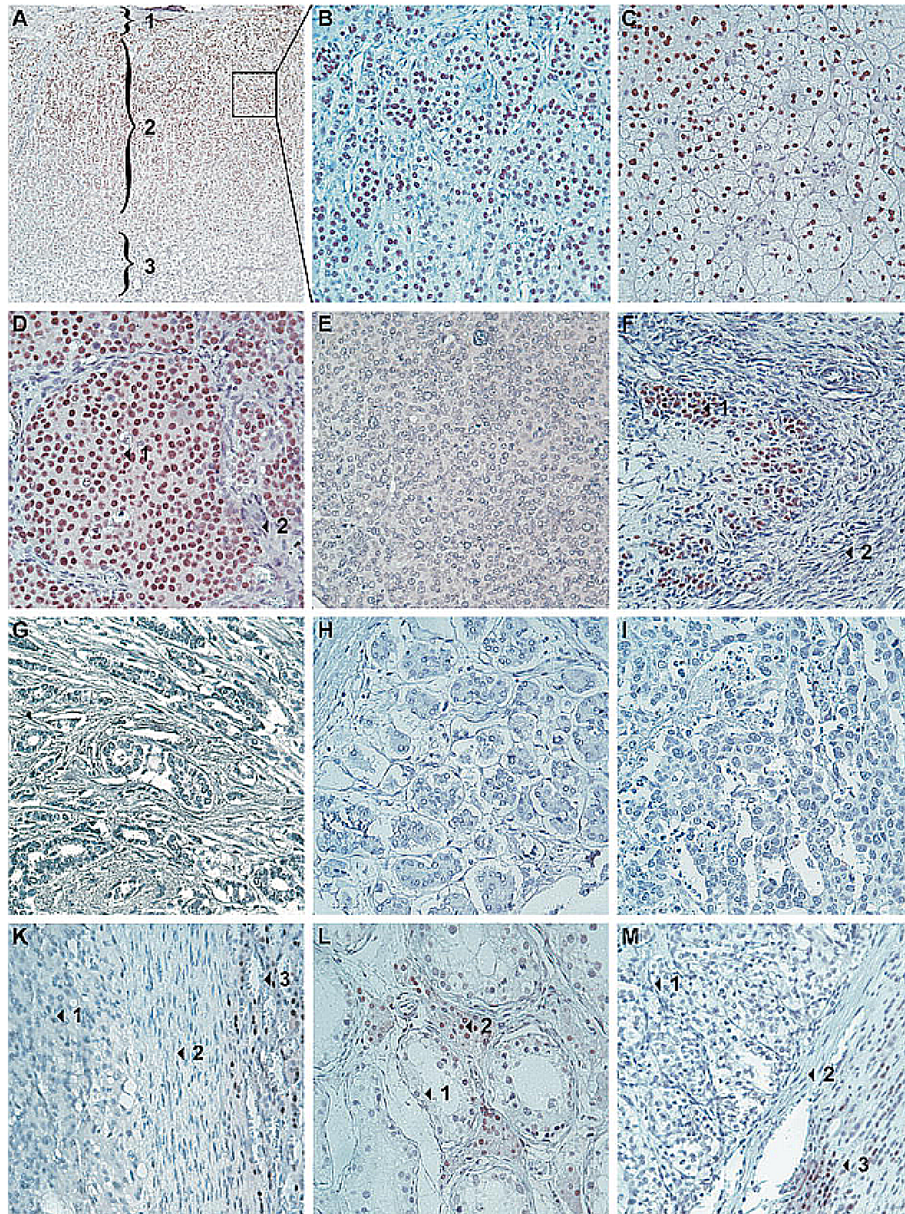

Figure 5.1: SF1 protein staining in normal, benign, and malignant tissues of steroidogenic and non-steroidogenic origin. A: normal adrenal gland (A1: capsule. A2: cortical zones. A3: medulla). Magnification: 100fold. B: magnification of indicated area of A2. C: adrenocortical adenoma. D (high SF1 protein staining. D1: tumor cells. D2: fibroblasts) and E (negative SF1 staining): adrenocortical carcinoma. F: normal ovary (F1: steroidogenic active theca and granulosa cells. F2: other cell types). G: breast carcinoma. H: pancreas carcinoma. I: endometrium carcinoma. K: pheochromocytoma (K1: medulla. K2: adrenocortical capsule. K3: steroidogenic active normal adrenocortical gland cells). L: intratubular germ cell neoplasia (L1: SF1 protein negative tumor cells. L2: steroidogenic active Leydig cells). M: renal clear cell carcinoma (M1: SF1 protein negative tumor cells. M2: adrenocortical capsule. M3: SF1 protein positive adrenocortical cells). Magnification B–M: 400fold. Figure taken from Sbiera et al. [2010].

Table 5.2: *SF-1* expression score distribution in steroidogenic and non-steroidogenic tissues.

		SF1 (Staining) (H-score)			
		Low			High
Tissue	n	0	0.1–1	1.5–2	3
NBST[a]	64	0 (0 %)	26 (41 %)		38 (59 %)
Normal adrenal gland[b]	6	0	1	2	3
Normal ovary	6	0	0	6	0
Endocrine-inactive adenoma	10	0	0	2	8
Aldosterone-producing adenoma	26	0	0	9	17
Cortisol-producing adenoma	16	0	0	6	10
Adrenocortical carcinoma	161	3 (2 %)	109 (68 %)		49 (30 %)
Primary	130	3	42	42	43
Local recurrences	18	0	9	5	4
Distant metastases	13	0	4	7	2
Non-steroidogenic tumors	73	73 (100 %)	0 (0 %)		0 (0 %)
Breast carcinoma	8	8	0	0	0
Colon carcinoma	7	7	0	0	0
Hepatocellular carcinoma	7	7	0	0	0
Endometrial carcinoma	2	2	0	0	0
Melanoma metastases	3	3	0	0	0
Non-Hodgkin lymphoma	2	2	0	0	0
Non small cell lung carcinoma	11	11	0	0	0
Ovarian carcinoma	4	4	0	0	0
Pancreatic carcinoma	5	5	0	0	0
Pheochromocytoma	8	8	0	0	0
Prostate carcinoma	4	4	0	0	0
Renal cell carcinoma	11	11	0	0	0
Seminoma[c]	1	1	0	0	0
Small cell lung carcinoma	1	1	0	0	0

[a]Normal and benign steroidogenic tissue.
[b]Only cells of *zonae reticularis, fasciculata, glomerulosa* were positive, whereas *medulla* cells were negative.
[c]Only normal Leydig cells showed positive staining but tumor cells were negative.

Table 5.3: *SF1* expression scores with associated patients characteristics. Table adapted from Sbiera et al. [2010].

	SF1 (n) [H-score]		
	Negative (3) [0]	Low (84) [0.1–2]	High (42) [3]
Age[a]	35 (25)	50 (16)	49 (17)
Sex			
Male (n=47)	0	35	12
Female (n=83)	3	49	31
Tumor stage[b]			
I–II (n=52)	2	36	14
III (n=39)	0	23	16
IV (n=33)	1	20	12
Hormone secretion[c]			
Non-secreting (n=17)	0	12	5
Glucocorticoids (n=48)	1	24	23
Sex-hormones (n=13)	1	10	2
Mineralcorticoids (n=6)	0	3	3

[a]Mean (standard deviation).
[b]In 6 cases tumor stage was not determined.
[c]In 46 patients no preoperative endocrine work-up was performed or no information on hormonal secretion was available.

5.2.2 FATE1 protein staining

FATE1 protein staining in immunohistochemistry was regionally distributed in the tumor and mostly cytoplasmic (Figure 5.2), as expected from recent publications [Yang et al., 2005]. Immunohistochemical staining was observed in overall 52 (84 %) samples presenting low and 10 (16 %) samples presenting high FATE1 staining of normal and benign steroidogenic tissue samples (Table 5.4 on page 45). From the low staining tissues, all 5 normal adrenal glands were negative as well as 5 of 7 hyperplasias, 8 of 9 endocrine-inactice adenomas, and 25 of 26 aldosterone-producing adenomas. As 'outlier' might be considered the subgroup of cortisol-producing adenomas as only 8 of 15 samples presented high FATE1 expression scores while the remaining 7 cases were negative for FATE1.

From the adrenocortical carcinoma group 86 samples (61 %) showed low and 55 samples (39 %) high FATE1 staining results (Fig. 5.2, and Table 5.4). 63 primary tumors, 11 local recurrences and 5 distant metastases were negative for FATE1. High staining was observed in 44 primary carcinomas, 5 local recurrences and 6 distant metastases. For statistical reasons the negative adrenocortical carcinoma samples were included in the H-score group encompassing 0.5–1 and together designated as low expression.

All 9 non-steroidogenic tumors we examined, i.e. colon, ovarian, pancreatic, and prostate carcinomas (each n=2) as well as a pheochromocytoma, revealed no FATE1 immunoreactivity (Table 5.4).

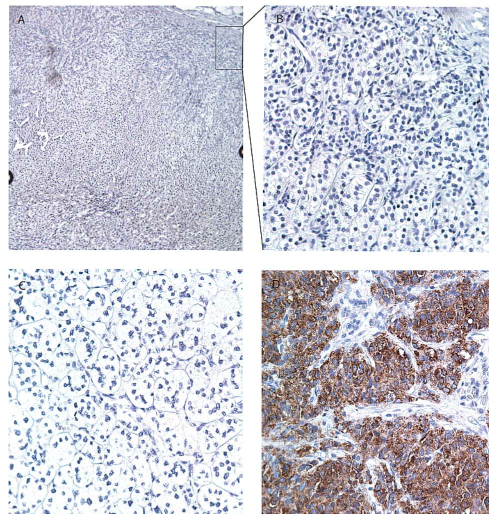

Figure 5.2: FATE1 protein staining in normal, benign, and malignant tissues of steroidogenic origin. A: normal adrenal gland. Magnification: 100fold. B: magnification of indicated area of A. C: adrenocortical adenoma. D: adrenocortical carcinoma. Magnification B–D: 400fold.

Considering all tumor tissues derived from non-steroidogenic organ entities and ACC samples for analysis of FATE1 expression, sensitivity, specificity, positive predictive value, and negative predictive value were 61 %, 100 %, 100 %, and 14 %, respectively. However, the sample size of non-steroidogenic tissue was much lower than in the SF1 analysis (see Table 5.2 on page 43).

Table 5.4: *FATE1* expression score distribution in steroidogenic and non-steroidogenic tissues.

		FATE1 (Staining) (H-score)			
		Low		High	
Tissue	n	0	0.1–1	1.5–2	3
NBST[a]	62	52 (84 %)		10 (16 %)	
Normal adrenal gland	5	5	0	0	0
Hyperplasia	7	5	1	1	0
Endocrine-inactive adenoma	9	8	0	0	1
Aldosterone-producing adenoma	26	25	1	0	0
Cortisol-producing adenoma	15	7	0	3	5
Adrenocortical carcinoma	141	86 (61 %)		55 (39 %)	
Primary	112	63	5	9	35
Local recurrence	17	11	1	1	4
Distant metastases	12	5	1	0	6
Non-steroidogenic tumors	9	9 (100 %)		0 (0 %)	
Colon carcinoma	2	2	0	0	0
Ovarian carcioma	2	2	0	0	0
Pancreatic carcinoma	2	2	0	0	0
Pheochromocytoma	1	1	0	0	0
Prostate carcinoma	2	2	0	0	0

[a]Normal and benign steroidogenic tissue.

5.2.3 DAX1 protein staining

DAX1 protein was present in the nucleus of all 20 normal and benign steroidogenic tissues encompassing normal adrenal gland, as well as endocrine-inactive, aldosterone-producing, and cortisol-producing adrenal adenoma (Figure 5.3 and Table 5.5 on page 46).

55 adrenocortical carcinoma samples (44 %) were DAX1-negative (38 primary tumors, 7 local recurrences, 12 distant metastases) and 71 (56 %) ACC samples (61 primary carcinomas, 8 local recurrences, 2 distant metastases) showed positive DAX1 immunoreactivity (Table 5.5). Interestingly, 61 primary ACC tumors and only 2 distant metastases showed positive DAX1 staining. For statistical reasons all samples presenting DAX1 immunoreactivity were grouped into positive staining.

In non-steroidogenic tumors DAX1 was present in all carcinomas of the colon and prostate (n=2, respectively); both negative and positive staining was observed in each, ovarian and pancreatic carcinoma (n=2, respectively; Table 5.5).

Considering all tumor tissues derived from non-steroidogenic organ entities and ACC samples for analysis of DAX1 expression, sensitivity, specificity, positive predictive value, and negative predictive value were 56 %, 25 %, 92 %, and 4 %, respectively. Again, sample size of non-steroidogenic tissue samples was much lower than in the SF1 analysis (see Table 5.2 on page 43).

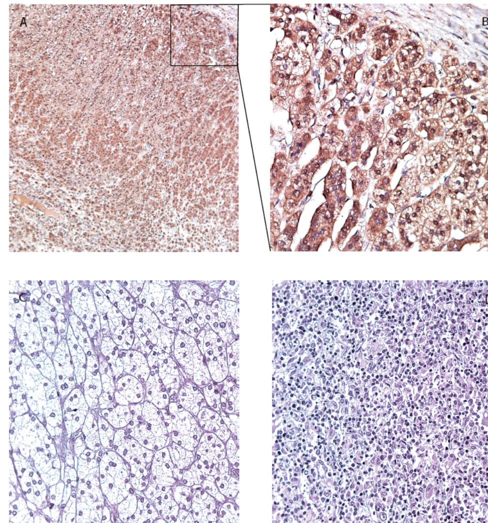

Figure 5.3: DAX1 protein staining in normal, benign, and malignant tissues of steroidogenic origin. A: normal adrenal gland. Magnification: 100fold. B: magnification of indicated area of A. C: adrenocortical adenoma. D: adrenocortical carcinoma. Magnification B–D: 400fold.

Table 5.5: *DAX1* expression score distribution in steroidogenic and non-steroidogenic tissues.

		DAX1 (Staining) (H-score)			
		Negative	Positive		
Tissue	n	0	0.1–1	1.5–2	3
NBST[a]	20	0 (0 %)	20 (100 %)		
Normal adrenal gland	5	0	0	1	4
Hyperplasia	-	-	-	-	-
Endocrine-inactive adenoma	4	0	1	0	3
Aldosterone-producing adenoma	6	0	1	5	0
Cortisol-producing adenoma	5	0	0	4	1
Adrenocortical carcinoma	126	55 (44 %)	71 (56 %)		
Primary	99	38	28	22	11
Local recurrence	15	7	3	3	2
Distant metastases	12	10	1	1	0
Non-steroidogenic tumors	8	2 (25 %)	6 (75 %)		
Colon carcinoma	2	0	0	0	2
Ovarian carcinoma	2	1	1	0	0
Pancreatic carcinoma	2	1	0	1	0
Pheochromocytoma	-	-	-	-	-
Prostate carcinoma	2	0	0	1	1

[a]Normal and benign steroidogenic tissue.

5.3 Survival analysis considering SF1, FATE1 and DAX1 protein expression in adrenocortical carcinoma

5.3.1 Univariate and multivariate analysis of SF1, FATE1, and DAX1 protein expression and their combinations in ACC influencing overall survival

SF1 protein staining was absent in only three patients. Therefore, the 3 samples were grouped with the patients presenting low staining intensity for survival analysis (in total n=87). Strong immunoreactivity was observed in 43 samples.

Within the high *SF1* expression group, 37 (86 %) out of 43 patients died of ACC. Within the group characterized by low SF1 protein staining intensity, only 43 (51 %) out of 84 patients died due to adrenocortical carcinoma.

Significant association between high *SF1* expression and mortality was observed performing univariate analysis (HR_{death} = 2.32 (95 % CI = 1.48–3.63); Table 5.6 on page 48) as median survival of patients presenting strong SF1 staining was 14 months (95 % CI = 9.9–18.1 months) whereas median survival of the low SF1 protein expression group was 49.8 months (95 % CI = 5.96–93.7 months; P = 0.002) [Sbiera et al., 2010].

Multivariate analysis adjusted for tumor stage, sex, and age did not modify the prognostic value of SF1 (HR_{death} = 2.45 (95 % CI = 1.54–3.89) presenting strong significance ($P < 0.001$), highlighting SF1s' prognostic value for patients suffering ACC (Table 5.6). Plotted overall survival is shown in Figure 5.4.

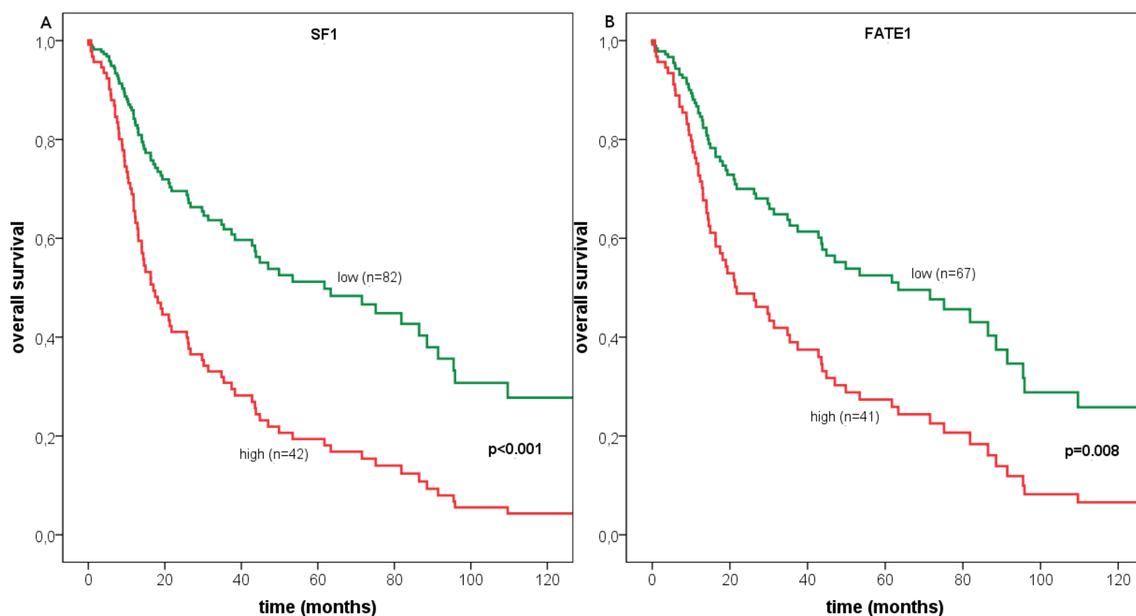

Figure 5.4: Plotted multivariate analysis of overall survival depending of SF1 and FATE1. A: overall survival depending of SF1 (HR = 2.45). B: overall survival depending of FATE1 (HR = 2.01).

Univariate analyses for each single FATE1 and DAX1 proteins revealed a strong correlation of high expression with shortened survival (FATE1: HR_{death} = 2.20; 95 % CI = 1.34–3.59, and DAX1: HR_{death} = 1.71; 95 % CI = 0.999–2.94, respectively (Table 5.6 on page 48)).

Table 5.6: Factors influencing overall survival according to univariate and multivariate analyses in patients with ACC.

	Univariate analysis			Multivariate analysis[a]		
Variables	HR	95 % CI	*P*	HR	95 % CI	*P*
Age[b]	1.00	0.99–1.02	0.509	1.01	0.99–1.02	0.340
Sex						
Male[c] (n=47)						
Female (n=83)	0.90	0.57–1.40	0.627	0.57	0.31–1.01	0.055
Tumor stage						
I–II[d] (n=53)						
III (n=40)	1.62	0.92–2.83	0.089	1.81	0.90–3.65	0.094
IV (n=34)	3.94	2.25–6.88	< 0.001	4.37	2.15–8.87	< 0.001
SF1						
Neg. & low[e] (n=83)						
High[f] (n=43)	2.32	1.48–3.63	< 0.001	2.45	1.54–3.89	< 0.001
FATE1						
Low[g] (n=68)						
High[h] (n=41)	2.20	1.34–3.59	0.002	2.01	1.20–3.56	0.008
DAX1						
Neg.[i] (n=36)						
High[j] (n=60)	1.71	0.999–2.94	0.05	1.46	0.84–2.53	0.175
SF1/FATE1						
Low[k] (n=51)						
High[l] (n=55)	2.63	1.56–4.41	< 0.001	2.76	1.62–4.71	< 0.001
SF1/DAX1						
Low[m] (n=53)						
High[n] (n=43)	1.89	1.14–3.16	0.014	1.75	1.05–2.93	0.033
SF1/FATE1/DAX1						
Low[o] (n=36)						
High[p] (n=49)	1.79	1.01–3.16	0.046	1.96	1.10–3.49	0.023

[a] Multivariate analysis was adjusted for age, ENSAT stage, and sex.
[b] HR of age is associated with one unit increase the predictor.
[c] Male sex was taken as reference category.
[d] Stage I–II was the reference category.
[e] Composed of H-score 0–2.
[f] Composed of H-score 3.
[g] Composed of H-score 0–1.
[h] Composed of H-score 1.5–3.
[i] Composed of H-score 0.
[j] Composed of H-score 1–3.
[k] SF1 neg. & low and FATE1 low.
[l] All other combinations.
[m] SF1 neg. & low and DAX1 neg.
[n] All other combinations.
[o] SF1/FATE1 low and SF1/DAX1 low.
[p] All other combinations.

Median survival of the low FATE1 protein expression group was 71.5 months *vs.* 19 months of the high protein expression group (95 % CI = 3.15–4.39; P = 0.0021). Patients within the DAX1-protein negative expression group were characterized by median survival of 71.5 months *vs.* 21.3 months in the high DAX1 protein expression group (95 % CI = 2.78–3.94; P = 0.0578). Combinations of SF1/FATE1, SF1/DAX1, and SF1/FATE1/DAX1 in univariate analysis (each low *vs.* high staining) revealed a strong significant correlation of $HR_{death} = 2.63$ (95 % CI = 1.56–4.41; $P < 0.001$) for the combination of SF1/FATE1. Significant correlations with shortened survival of SF1/DAX1 ($HR_{death} = 1.89$; 95 % CI = 1.14–3.16; $P = 0.014$) and

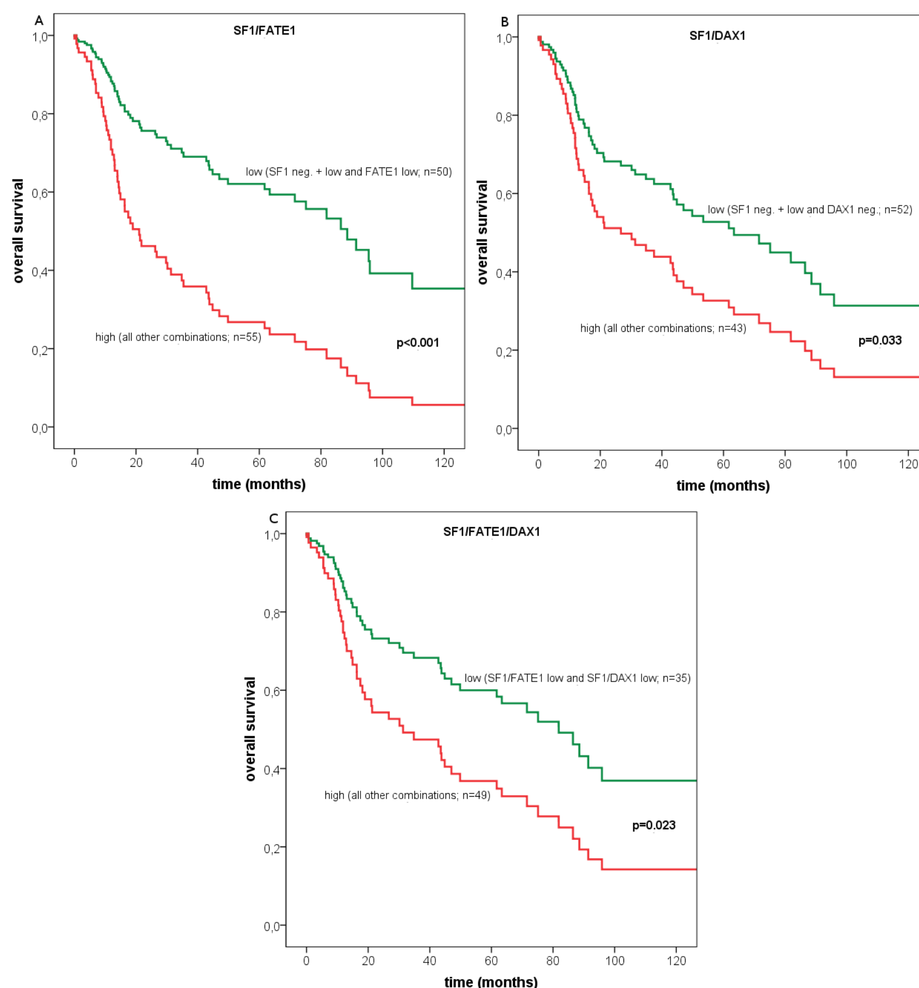

Figure 5.5: Plotted multivariate analysis of overall survival depending of combinations of SF1, FATE1, and DAX1 protein staining. A: overall survival depending of combination of SF1 and FATE1 protein staining (HR = 2.76). B: overall survival depending of combining SF1 with DAX1 protein expression (HR = 1.75). C: overall survival depending of combination of the three proteins SF1, FATE1, and DAX1 (HR = 1.96).

SF1/FATE1/DAX1 (HR_{death} = 1.79; 95 % CI = 1.01–3.16; P = 0.046) were also observed (Table 5.6 on page 48).

FATE1 protein staining had still a strong correlation with overall survival in multivariate setting (HR_{death} = 2.01; 95 % CI = 1.20–3.56; P = 0.008; Table 5.6 on page 48, and Figure 5.4), whereas DAX1 immunoreactivity showed no change in overall survival in multivariate setting (HR_{death} = 1.46; 95 % CI = 0.84–2.53; P = 0.175; Table 5.6 on page 48). Multivariate analysis combining immunohistochemical results of SF1 and FATE1 (SF1/FATE1) revealed strong significant correlation on overall survival (HR_{death} = 2.76; 95 % CI = 1.62–4.71; $P <$ 0.001; Table 5.6 on page 48 and Figure 5.5 A on page 49). Combining SF1 and DAX1 protein expression data revealed significant correlation with multivariate patient survival (HR_{death} = 1.75; 95 % CI = 1.05–2.93; P = 0.033; Table 5.6 on page 48 and Figure 5.5 B). Significant correlation of overall survival was observed if all three protein expressions, i.e. SF1, FATE1, and DAX1, were combined in a multivariate setting (HR_{death} = 1.96; 95 % CI = 1.10–3.49; P = 0.023; Table 5.6 on page 48 and Figure 5.5 C).

5.3.2 Univariate and multivariate analysis considering SF1, FATE1, and DAX1 protein expression and their combinations influencing disease-free survival after complete tumor resection

Investigating patient survival after complete tumor resection (n=48) revealed that high *SF1* expression showed a strong significant shorter median recurrence-free survival (8.8 months; 95 % CI = 7.7–9.9) *vs.* 37.7 months (95 % CI = 9.8–65.6; $P < 0.001$) [Sbiera et al., 2010]. Within the high *SF1* expression group 39 patients (90 %) out of total 43 and 50 (59 %) of 84 patients of the low SF1 expression group showed a recurrence.

Univariate analysis of *SF1* expression presented a strong significant correlation with recurrence-free survival ($HR_{recurrence} = 3.75$; 95 % CI = 1.72–8.20; $P = 0.001$; Table 5.7 on page 51). FATE1 protein expression showed no correlation with patients recurrence-free survival ($HR_{recurrence} = 1.13$; 95 % CI = 0.48–2.64; $P = 0.782$) as well as DAX1 ($HR_{recurrence} = 1.64$; 95 % CI = 0.69–3.92; $P = 0.267$) in univariate setting (Table 5.7 on page 51). Median survival for FATE1 low expression group *vs.* high expression group was 25.5 months *vs.* 15.2 months, respectively (95 % CI = 1.250–2.106; $P = 0.7817$). Median disease-free survival for DAX1 negative *vs.* high expression group was 37.7 months *vs.* 18.4 months, respectively (95 % CI = 0.267–1.433; $P = 0.2627$). Whereas the combination of SF1 and DAX1 (SF1/DAX1; $HR_{recurrence} = 2.10$; 95 % CI = 0.90–4.88; $P = 0.085$) and SF1 combined with FATE1 and DAX1 showed only a trend for recurrence-free survival (SF1/FATE1/DAX1; $HR_{recurrence} = 1.67$; 95 % CI = 0.64–4.33; $P = 0.292$) the combination of SF1 and FATE1 (SF1/FATE1) showed significant correlation ($HR_{recurrence} = 2.38$; 95 % CI = 1.03–5.51; $P = 0.043$) in univariate analysis (Table 5.7).

Of note, the prognostic significance of *SF1* expression was confirmed by multivariate analysis of recurrence-free survival ($HR_{recurrence} = 3.79$; 95 % CI = 1.66–8.68; $P = 0.002$) which is shown in Figure 5.6 A, and Table 5.7 on page 51.

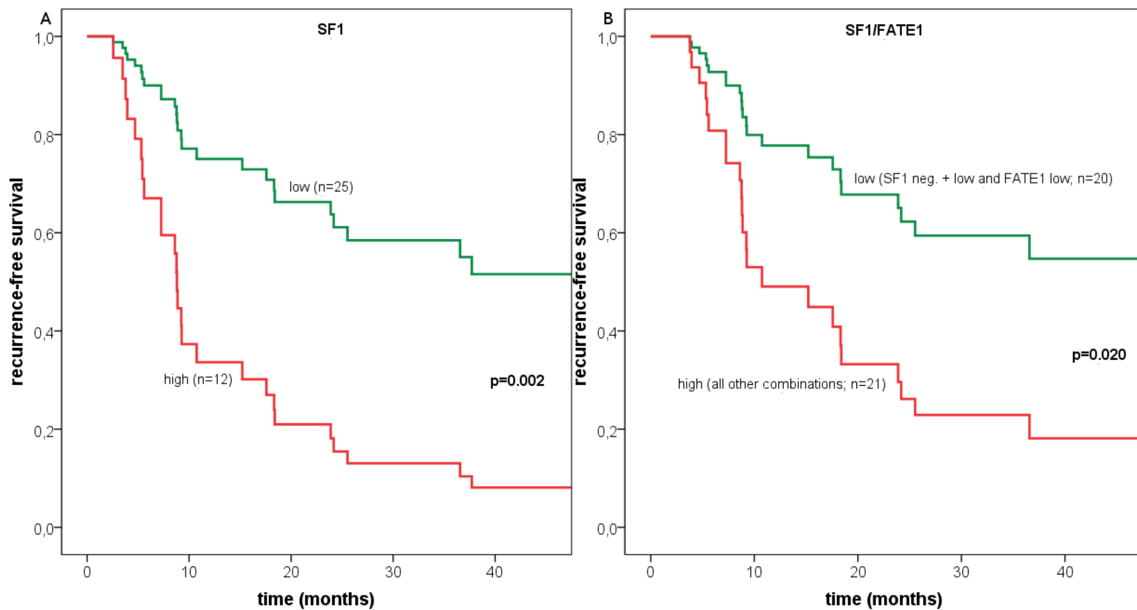

Figure 5.6: Multivariate analysis of SF1 protein and SF1/FATE1 protein combination in dependence of recurrence-free survival. A: recurrence-free survival in dependence of SF1 (HR = 3.79). B: recurrence-free survival in dependence of the combination of SF1 and FATE1 (HR = 2.83).

Table 5.7: Factors influencing recurrence-free survival according to univariate and multivariate analysis in patients with ACC.

	Univariate analysis			Multivariate analysis[a]		
Variables	HR	95 % CI	*P*	HR	95 % CI	*P*
Age[b]	0.98	0.96–1.00	0.167	0.98	0.95–1.00	0.084
Sex						
Male[c] (n=17)						
Female (n=31)	0.71	0.34–1.51	0.380	0.65	0.30–1.41	0.275
Tumor stage						
I–II[d] (n=31)						
III (n=17)	1.48	0.70–3.10	0.306	1.40	0.62–3.14	0.419
SF1						
Neg. & low[e] (n=35)						
High[f] (n=12)	3.75	1.72–8.20	0.001	3.79	1.66–8.68	0.002
FATE1						
Low[g] (n=27)						
High[h] (n=14)	1.13	0.48–2.64	0.782	1.34	0.56–3.14	0.517
DAX1						
Neg.[i] (n=16)						
High[j] (n=22)	1.64	0.69–3.92	0.267	2.04	0.76–5.48	0.156
SF1/FATE1						
Low[k] (n=20)						
High[l] (n=21)	2.38	1.03–5.51	0.043	2.83	1.18–6.82	0.02
SF1/DAX1						
Low[m] (n=25)						
High[n] (n=13)	2.10	0.90–4.88	0.085	2.15	0.85–5.46	0.107
SF1/FATE1/DAX1						
Low[o] (n=15)						
High[p] (n=19)	1.67	0.64–4.33	0.292	1.68	0.61–4.68	0.318

[a]Multivariate analysis was adjusted for age, ENSAT stage and sex.
[b]HR of age is associated with one unit increase the predictor.
[c]Male sex was taken as reference category.
[d]Stage I–II was the reference category.
[e]Composed of H-score 0–2.
[f]Composed of H-score 3.
[g]Composed of H-score 0–1.
[h]Composed of H-score 1.5–3.
[i]Composed of H-score 0.
[j]Composed of H-score 1–3.
[k]SF1 neg. & low and FATE1 low.
[l]All other combinations.
[m]SF1 neg. & low and DAX1 neg.
[n]All other combinations.
[o]SF1/FATE1 low and SF1/DAX1 low.
[p]All other combinations.

Furthermore, no influence was observed investigating single protein expression of FATE1 and DAX1 in multivariate setting ($HR_{recurrence}$ = 1.34; 95 % CI = 0.56–3.14; P = 0.517 and $HR_{recurrence}$ = 2.04; 95 % CI = 0.76–5.48; P = 0.156, respectively; Table 5.7 on page 51). As observed in univariate analysis, multivariate analysis revealed the same significant correlation with recurrence-free survival of combined *SF1/FATE1* expression scores ($HR_{recurrence}$ = 2.83; 95 % CI = 1.18–6.82; P = 0.02), while SF1/DAX1 and SF1/FATE1/DAX1 combinations showed no significant correlation ($HR_{recurrence}$ = 2.15; 95 % CI = 0.85–5.46; P = 0.107 and $HR_{recurrence}$ = 1.68; 95 % CI = 0.61–4.68; P = 0.318; Table 5.7).

5.4 *SF1* gene copy number investigation and correlation with SF1 protein expression

Evaluation of 5 normal adrenal glands revealed normal *SF1* gene copy number — which was also the case for the 2 aldosterone-producing, 2 cortisol-producing, 1 endocrine-inactive adenoma and 1 hyperplasia. Interestingly, pleomorphic nuclei presenting *SF1* polyploidy were observed in 1 cortisol-producing, the 1 endocrine-inactive adenoma and 1 hyperplasia (data not shown). This was despite the fact that H-scores for SF1 protein expression in normal adrenals were quite variable (1 x 1, 2 x 2 and 2 x 3). Both aldosterone-producing and cortisol-producing adenomas presented each H-scores of 2 and 3. The endocrine-inactive adenoma and hyperplasia had both an H-score of 3.

Of in total 20 primary adrenocortical carcinomas 1 (5 %) had only 1 *SF1* gene copy, 11 cases (55 %) presented 2 *SF1* gene copies, 7 primary ACC (35 %) were characterized by increased *SF1* gene copies (3 specific FISH signals), and 1 (5 %) showed an amplification of the *SF1* gene. The investigated local recurrence presented normal *SF1* gene copy number. Its corresponding malignant primary tumor showed three copies of *SF1*. Both metastases were characzterized by normal gene copy numbers of *SF1* and their corresponding primary carcinomas presented normal gene copy number and a gain of *SF1*. Figure 5.7 shows examples of normal gene copy number (Figure 5.7 B), loss and gain of *SF1* presenting 1 and 3 fluorescent signals, respectively (Figure 5.7 A and C), and amplification of the *SF1* gene (Figure 5.7 D).

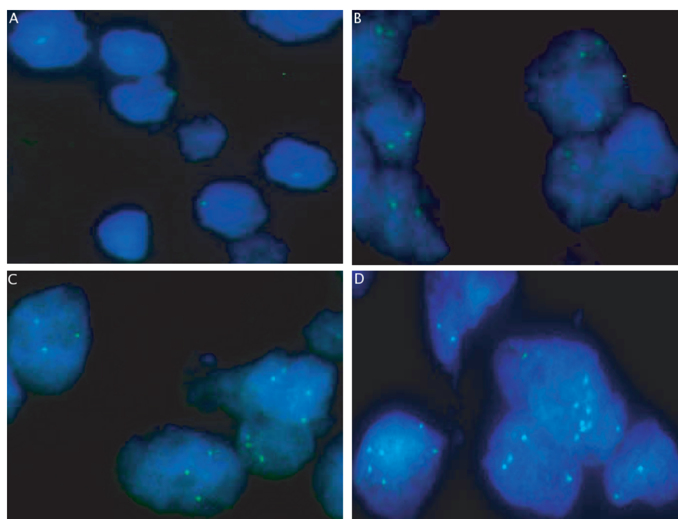

Figure 5.7: *SF1* gene copy number in primary adrenocortical carcinoma. A: 1 *SF1* gene copy number. B: normal *SF1* gene copy number. C: chromosomal gain presenting 3 *SF1* gene copy numbers. D: amplification of the *SF1* gene.

Correlation of SF1 protein expression and *SF1* gene copy number in adrenocortical carcinoma revealed that *i.)* 1 out of 3 SF1 negative samples (33 %) showed 3 gene copies of *SF1* (all primary tumors), *ii.)* 3 samples (30 %) out of 10 adrenocortical carcinoma (7 primary ACC, the local recurrence and both metastases) characterized by H-score 1 presented 3 *SF1* gene copies (all primary tumors), *iii.)* of the 6 ACC cases within the H-score 2 group (all primary tumors) 1 (17 %) presented 1 *SF1* gene copy, 2 samples (33 %) presented 3 gene copies, and 1 case (17 %) presented *SF1* gene amplification, and *iv.)* out of 4 samples (all

primary carcinomas) with H-score of 3 1 case (25 %) presented 3 gene copy numbers of *SF1* (Figure 5.8).

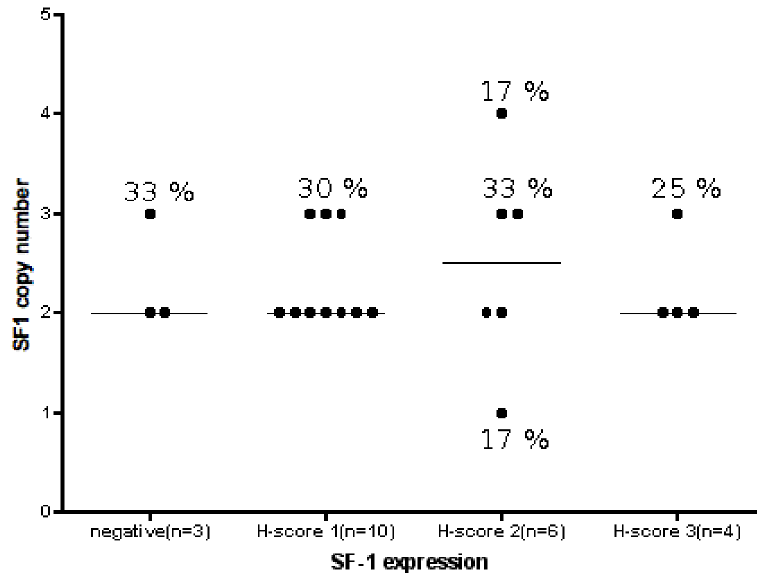

Figure 5.8: Correlation of *SF1* gene copy number with SF1 protein expression in adrenocortical carcinoma. Each dot represents one sample. Percentages of samples showing aberrantl gene copy numbers of *SF1* are given. Tissues were comprised of 20 primary adrenocortical carcinoma, 1 local recurrence and 2 metastases. The local recurrence and both metastases had an H-score of 1.

—Absence of evidence isn't evidence of absence.

D. T. Max, The Family That Couldn't Sleep

—We must delve into the properties of proteins, we must learn the structure of colloidal organic systems, of enzymes, of protoplasmic organisation, etc. The road ahead is hard and long but without doubt it leads to the ultimate knowledge of the nature of life.

A. I. Oparin, The Origin of Life

Discussion

6.1 Diagnostic value of SF1, FATE1, and DAX1 in adrenocortical carcinoma

Due to the heterogeneity and rareness of ACC, definite histological diagnosis is often difficult. Moreover, the histomorphological criteria used for discrimination of pheochromocytoma or metastasis from other (retroperitoneal) tumors are not well established [Saeger, 2000, Saeger et al., 2003, Sasano et al., 2006, Volante et al., 2008]. To overcome this limitation we looked for highly specific diagnostic markers for differentiation of benign and malignant adrenocortical tumors and other retroperitoneal tumors. We focused on the proteins SF1, FATE1, and DAX1.

6.1.1 Is SF1 protein expression useful as a diagnostic tool?

Evaluation of 161 ACC samples revealed SF1 as a well suited immunohistochemical marker for ACC. This is based on the observation that only 2 % of all ACC were SF1 negative (see Table 5.2 on page 43). SF1-positivity was also observed in all 52 adrenocortical adenomas (see Table 5.2). Since both benign and malignant adrenocortical tumors were positive for SF1, it is obviously not a useful marker for differentiation of adenomas and carcinomas of the adrenal gland. However, all 73 non-steroidogenic tumors showed no immunohistochemical verifiable SF1 protein expression (see Table 5.2 on page 43), except steroidogenic active non-tumor cells (e.g. Leydig cells of the seminoma and granulosa and theca cells of nomal ovary). This observation underlines SF1s higher sensitivity (98.6 %) for determining an adrenocortical origin of adrenal lesion than D11, melan-A, and inhibin-α which show a sensitivity of 73 %, 72 %, and 69 %, respectively (see Table 3.2 on page 22). Specificity is comparable to the other three markers (each 99 %) and SF1 (100 %). Furthermore, SF1 is superior in positive and negative predictive value (100 % and 97.3 %, respectively) than D11 (98 % and 90 %), melan-A (90 % and 97 %), and inhibin-α (93 % and 95 %). Recently, Sangoi and McKenney [2010] reported 87 % and 86 % of SF1 staining intensity 1 and $\geqq$ 2+, respectively, in adrenocortical tissues comprised of 3 adrenal rests, 6 hyperplasias, 4 neoplasms of uncertain malignant potential, 43 adenomas, and 7 carcinomas, further emphasizing its usage as diagnostic tool. Already in 1995, Sasano et al. [1995c] suggested SF1 as a useful marker in the differential diagnosis of ACC, but only the results of 17 ACC samples stained with SF1 antibody were published until 2009 [Sasano et al., 1995a,b, Kaneko et al., 2008].

2 of the 3 SF1-negative ACC were hormonally active tumors (see Table 5.3 on page 43). Considering that SF1 is involved in steroidogenesis this inconsistency might be rooted in methodological limitations of immunohistochemistry using formalin-fixed, paraffin-embedded tissues. Since no frozen tissue of the 3 SF1 negative cases was available we were not able to

investigate expression at the mRNA level (to draw conclusions about the SF1 protein level). An alternative explanation could be that these adrenocortical carcinomas are truly negative for SF1. If this is the case, activating mutations of SF1 and/or downstream pathway targets could be hypothesized.

Sasano et al. [1996] showed that SF1 was observed only in intratumoral stromal cells of ovarian tumors which is congruent with negative IHC results of our examined ovarian carcinomas. Negative staining results of investigated tissues of renal, liver, and lung carcinomas, are in line with results published by Sasano et al. [1995c]. Absence of SF1 protein staining in pheochromocytoma is also found in the literature [Sangoi and McKenney, 2010]. Negative SF1 protein IHC results in breast carcinoma confirmed RT-PCR results published by Chen et al. [1999], which is also the case for endometrial carcinoma using immunohistochemistry and reverse transcription and real-time PCR [Saito et al., 2005].

Methodological correctness is further supported by the finding that only steroidogenic cells (Leydig, theka, and granulosa cells) embedded in non-steroidogenic tumors were shown to express *SF1* in otherwise SF1 protein negative tumors [Takayama et al., 1995].

6.1.2 Can the diagnostic accuracy be improved by FATE1 or DAX1?

In this work, we have shown that except 3 cases all adrenocortical carcinoma samples stained positive for SF1. Since all adrenocortical adenomas were also characterized by SF1-positive immunohistochemical results, we chose to investigate the potential role of two genes harbouring a SF1 responsive element, namely FATE1 and DAX1, to improve the diagnostic accuracy of SF1 with the hypothesis that the down-stream signaling in the two tumor entities is different.

FATE1 10 samples (16 %) comprised of normal adrenocortical tissues and adenomas and 55 cases (39 %) of ACC showed high staining for FATE1 (see Table 5.4 on page 45). All 9 non-steroidogenic tumors showed no FATE1-specific immunohistochemical reaction (see Table 5.4). Considering FATE1-specific sensitivity, specificity, positive and negative predictive value (61 %, 100 %, 100 %, and 14 %, respectively) it is reasonable to conclude that *i.)* FATE1 is no valuable diagnostic tool for adrenocortical carcinoma or differentiation of benign and malignant adrenal lesions, and *ii.)* FATE1 is no valuable tool for discriminating adrenocortical and non-steroidogenic tumors.

It is important to state that sample size of non-adrenocortical tissues is in total 9 so that no final statement about protein expression in these tumors is possible. This can explain the low sensitivity and negative predictive value of FATE1 to some extent, since they are dependent on tissues considered/expected negative for the protein of interest.

The report of absense of *FATE1* transcription products in normal adrenal glands [Dong et al., 2003] is immunohistochemically confirmed by our study. Dong et al. [2003] published that *FATE1* mRNA was found in malignant tumor tissues of liver, colon, and gastric cancer. Discrepancy concerning our FATE1 protein negative colon carcinomas and *FATE1* mRNA positive malignant colon tissues reported by Dong et al. [2003] might be rooted in the fact that immunohistochemistry is not as sensitive as RT-PCR and real-time PCR.

DAX1 DAX1 staining was observed in 71 samples (56 %) of all adrenocortical carcinomas and all 20 normal and benign steroidogenic tissues (normal adrenals, n=5, and benign adrenal adenomas, n=15) were DAX1 positive (see Table 5.5 on page 46). Interestingly, 6 samples

(75 %) of the non-steroidogenic tumors showed also DAX1-specific reactions in immunohistochemistry (see Table 5.5). These observations lead to the low diagnostic accuracy of DAX1 with a sensitivity, specificity, positive and negative predictive value of 56 %, 25 %, 92 %, and 4 %, respectively, clearly indicating that DAX1 is no diagnostic tool for *i.)* differentiation of ACC and other (retroperitoneal) tumors, and *ii.)* differentiation of adrenocortical adenomas and carcinomas. Interestingly, only 2 of a total of 10 distant metastases showed DAX1 immunoreactivity which is in contrast to findings published by Kaneko et al. [2008], where tissue from four patients with advanced and metastatic adrenocortical carcinoma stained positive.

Although we did not consider the hormonal status of our ACC patient cohort, the observed positivity for the DAX1 protein in all normal and benign steroidogenic tissues (n=20; see Table 5.5) is contrary to findings by Reincke et al. [1998] who observed negative or low *DAX1* mRNA levels in 11 aldosterone-producing adenomas and 2 aldosterone-secreting ACC. Furthermore, Reincke et al. [1998] reported that 8 cortisol-producing adenoma were characterized by intermediate *DAX1* expression, whereas 3 nonfunctional adenomas showed high transcript levels of *DAX1*. Besides the low number of samples, the different techniques (immunohistochemistry investigating protein *vs.* Northern blotting investigating mRNA) might be responsible for the difference.

6.2 Prognostic value of SF1, FATE1, and DAX1 in adrenocortical carcinoma

Up to now, only a few prognostic markers have been established for ACC [Terzolo et al., 2001, Volante et al., 2006, Fenske et al., 2009, Ronchi et al., 2009]. Therefore, we considered the influence of the proteins SF1, FATE1, and DAX1 on overall and recurrence-free survival to evaluate their value in patients suffering ACC.

6.2.1 Is SF1 protein expression a useful prognostic tool?

Our analysis revealed that elevated SF1 protein expression correlates negatively with overall (uni-/multivariate $HR_{death} = 2.32$, $P < 0.001$ / $HR_{death} = 2.45$, $P < 0.001$; see Table 5.6 on page 48) and recurrence-free survival (uni-/multivariate $HR_{recurrence} = 3.75$, $P < 0.001$ / $HR_{recurrence} = 3.79$, $P < 0.002$; see Table 5.7 on page 51). Thus, our results are congruent with data showing an increased SF1 protein level associated with decreased apoptosis and increased proliferation of human adrenocortical cells *in vitro* as well as inducing cortical tumors of adrenals in mice [Doghman et al., 2007]. Further evidence for this observation comes from reports showing that inverse agonists of SF1 are able to inhibit proliferation of adrenocortical cancer cells [Doghman et al., 2009]. Very recently, it was shown that in adrenocortical adenomas inhibition of SF1 resulted in increased apoptosis of cells [Hu et al., 2010]. Moreover, in our study we included also a series from French colleagues in which high *SF1* mRNA levels are associated with poor prognosis in patients suffering adrenocortical carcinoma [Sbiera et al., 2010].

Importantly, even after adjustement for tumor stage in multivariate analysis (see Tables 5.6 and 5.7 on page 48 and 51, respectively) SF1 is still shown to correlate significantly with poor prognosis [Sbiera et al., 2010]. The definite role of SF1 in tumorigenesis and malignancy still needs to be investigated. It can be hypothesized that the cellular context of ACC resembles that of fetal adrenals [Sbiera et al., 2010]. The assumption is based on the fact that SF1 is

an important factor in adrenogenesis [Luo et al., 1994, Crawford et al., 1997, Val et al., 2003]. Involvement of SF1 in adrenogenesis could, at least in part, explain the paradox that *SF1* is equally highly expressed in benign and malignant adrenocortical tumors but shows correlation with survival only in ACC. This view is further supported by the observed lack of correlation between *SF1* expression and patients hormonal status [Sbiera et al., 2010]. Another aspect for the tumorigenetic capacities of SF1 comes from the report that *CXorf6* shows a binding site for SF1 and that its gene product is capable to transactivate the noncanonical NOTCH target gene *HES3* [Fukami et al., 2008]. From this observation, building a bridge to embryonic stem cells is easy, since it was shown that *HES3* is a target gene for embryonic stem cell-specific transcription factors [Katoh and Katoh, 2007], and that it contains a binding site of POU5F1 which is also positively regulated by SF1 [Yang et al., 2007]. As 'feasting the beast', i.e. cancer stem cells, although highly speculative, SF1 might be involved in cancer stem cell maintenance.

6.2.2 Can the prognostic accuracy be improved by FATE1 or DAX1?

As shown by our study, SF1 is of great prognostic value for adrenocortical carcinoma. Since we evaluated the H-score of the SF1 responsive element harbouring genes *FATE1* and *DAX1* in diagnosis, we can derive the impact of their protein expression on prognosis.

FATE1 Our work is the first evaluation of FATE1 protein by immunohistochemistry in adrenocortical carcinoma and its possible influence on patients overall and recurrence-free survival.

Univariate and multivariate analysis of FATE1 protein distribution scores revealed a significant correlation ($HR_{death} = 2.20$, $P = 0.002$ / $HR_{death} = 2.01$, $P = 0.008$; see Table 5.6 on page 48) on patient overall survival but not on recurrence-free survival (uni-/multivariate $HR_{recurrence} = 1.13$, $P = 0.782$ / $HR_{recurrence} = 1.34$, $P = 0.52$; see Table 5.7 on page 51). So, although FATE1 is no valuable diagnostic tool, it can be considered a prognostical tool for overall survival. It is tempting to speculate that other factors influence patient survival that play no role in recurrence-free survival and are unknown.

DAX1 Both univariate and multivariate analysis results presented here revealed a non-significant correlation of DAX1 scores and overall patient survival ($HR_{death} = 1.71$, $P = 0.05$ / $HR_{death} = 1.46$, $P = 0.18$; see Table 5.6 on page 48) or recurrence-free survival ($HR_{recurrence} = 1.64$, $P = 0.267$ / $HR_{recurrence} = 2.04$, $P = 0.156$; see Table 5.7 on page 51), but the high hazard ratios indicate that DAX1 might have some influence especially on recurrence-free survival.

Oda et al. [2009] published evidence that elevated levels of DAX1 were associated with higher lymph node metastases and recurrence in lung adenocarcinoma. Nakamura et al. [2009b] reported significant negative correlation of DAX1 immunoreactivity and Gleason score in prostate cancer. Saito et al. [2005] suggested an inhibitory role of DAX1 on cell proliferation and progression of endometrial carcinoma. Transferring results published by Saito et al. [2005] to adrenocortical carcinoma imply that the majority of ACC cases had to be DAX1 negative to circumvent its inhibitory role. Although negative and positive DAX1 staining groups showed an almost equal distribution in our study (44 % were DAX1 negative and 56 % DAX1 positive), almost all metastases were DAX1 protein negative which might support the hypothesis of Saito et al. [2005], from a tumor evolutionary point of view.

DAX1 antagonizes a number of transcription factors, like Wilms tumor 1 (WT1) [Lalli and Sassone-Corsi, 2003], which is capable to antagonize transcription of anti-apoptotic *B-cell CLL/lymphoma 2 (Bcl-2)* gene [Hewitt et al., 1995]. This implies, like discussed by Oda et al. [2009], that, if DAX1 inhibits WT1 protein action to repress the promoter of *Bcl-2*, cells are capable to circumvent programmed cell death. Since none of WT1 or Bcl-2 were investigated in our study, it is highly speculative comment on this hypothesis. It is only possible to highlight the observed relative high sample size (56 %) of DAX1-positive tissues and that the *DAX1* gene product showed no significant influence in overall and recurrence-free survival in univariate and multivariate settings.

In adrenal development, PBX1 is synergistically activated by SF1 and DAX1 [Ferraz-de Souza et al., 2009]. *PBX1*$^{-/-}$ mice are lacking adrenal glands [Schnabel et al., 2003] and haploinsufficient mice for *PBX1* are characterized by reduced adrenal weight [Lichtenauer et al., 2007]. As we suggested that the cellular context of ACC resembles the fetal phenotype [Sbiera et al., 2010], PBX1 might be a further promising target for prognosis. Alternatively, *β-catenin* expression could also be investigated due to a reported positive influence of *DAX1* gene transcription [Mizusaki et al., 2003]. Furthermore, an influence of DAX1-α may be explored due to its inability to repress SF1 [Hossain et al., 2004].

6.2.3 Are combinations of SF1, FATE1, and DAX1 useful to estimate patients' survival?

Since the expression of both FATE1 and DAX1 should at least show a trend towards correlation with clinical outcome we investigated the influence of the combinations SF1/FATE1, SF1/DAX1 and SF1/FATE1/DAX1 on overall and recurrence-free survival.

Only the combination SF1/FATE1 showed a significant correlation in recurrence-free survival (uni-/multivariate $HR_{recurrence} = 2.38$, $P = 0.043$ / $HR_{recurrence} = 2.83$, $P = 0.02$; see Table 5.7 on page 51). Moreover, strong significance was observed in overall survival settings (uni-/multivariate $HR_{death} = 2.63$, $P < 0.001$ / $HR_{death} = 2.76$, $P < 0.001$; see Table 5.6 on page 48). It is speculative to assume that further factors are involved in overall patient survival. This assumption is underlined by our observation that combinations SF1/DAX1 (uni-/multivariate $HR_{death} = 1.89$, $P = 0.014$ / $HR_{death} = 1.75$, $P < 0.033$; see Table 5.6) and SF1/FATE1/DAX1 (uni-/multivariate $HR_{death} = 1.79$, $P = 0.046$ / $HR_{death} = 1.96$, $P = 0.023$; see Table 5.6) showed significant correlation in overall survival. However, combinations of SF1/DAX1 (uni-/multivariate $HR_{recurrence} = 2.10$, $P = 0.085$ / $HR_{recurrence} = 2.15$, $P = 0.107$; see Table 5.7) and SF1/FATE1/DAX1 (uni-/multivariate $HR_{recurrence} = 1.67$, $P = 0.292$ / $HR_{recurrence} = 1.68$, $P = 0.318$; see Table 5.7) revealed no significant correlation in recurrence-free survival. Thus, it is recommendable to utilize the SF1/FATE1 combination for estimating overall and recurrence-free patient survival.

Although the combination of SF1/FATE1 presented similar strong significant correlation in both univariate and multivariate analyses as SF1 alone (see above), the hazard ratio of SF1/FATE1 is slightly higher in overall survival (uni-/multivariate 2.32 and 2.45 for SF1, and 2.63 and 2.76 for SF1/FATE1, respectively). In recurrence-free survival, hazard ratio of SF1 is higher than that of SF1/FATE1 (uni-/multivariate 3.75 and 3.79 for SF1, and 2.38 and 2.83 for SF1/FATE1, respectively). Thus, we strongly suggest SF1 alone as prognostic tool. SF1/FATE1 might be more appropriate in special cases but one has to consider that these investigations with additional antibodies are time-, tissue-, and money-consuming.

6.3 Is *SF1* gene amplification responsible for overexpression of the *SF1* gene?

Evaluation of *SF1* gene copy number in adult ACT was performed to investigate if SF1 protein overexpression is rooted in increased gene copies or amplification as it is the case in childhood ACT [Figueiredo et al., 2005, Pianovski et al., 2006, Almeida et al., 2010].

All investigated normal adrenal glands and adrenocortical adenoma presented normal *SF1* gene copy numbers. Interestingly, in adenoma, pleomorphic nuclei were observed. Pleomorphic nuclei in adenoma have already been observed in adenomas [Sasano et al., 1995a, Saeger, 1995]. We speculate that pleomorphic nuclei are due to polyploidy. Cibas et al. [1990] reported a significant correlation between ploidy and nuclear grade, which should not be surprising because — at least partly — nuclear grade can be used for rough visual estimations of deoxyribonucleic acid content. Of note, staging of nuclear grade in ACC is also dependent on nuclear size [Weiss, 1984, Weiss et al., 1989]. Recently, Gisselsson et al. [2001] suggested that abnormalities in nuclear shape might be an indicator of genetic instability, which is also in line with our observation that adenomas with pleomorphic nuclei presented polyploidy for *SF1* (data not shown) which adds further evidence to our hypothesis.

Our primary adrenocortical carcinoma cohort revealed that 11 of a total of 20 cases presented normal gene copy number of *SF1*, whereas only one sample showed loss of *SF1* and one case amplification (see section 5.4 on page 52). The remaining 7 samples had gains in *SF1* gene. The samples of local recurrence (n=1) and metastases (n=2) showed normal gene copy number of *SF1*. Aneuploidy, an abnormal number of chromosomes, is a well known observation in adrenocortical carcinoma [Klein et al., 1985, Bowlby et al., 1986, Hosaka et al., 1987, Cibas et al., 1990, Suzuki et al., 1992, Haak et al., 1993, Pignatelli et al., 1998, Blanes and Diaz-Cano, 2006]. Our study supports the observation to a certain degree — although the aforementioned experiments were mainly performed using flow cytometric analysis whereas our method-of-choice was fluorescence *in situ* hybridization explicitly targeting chromosome 9q33–9q34.

We report that adult adrenocortical carcinoma is also characterized by increased gene copy number of *SF1* like childhood ACC presenting gains of *SF1* [Figueiredo et al., 2005, Pianovski et al., 2006]. Concentrating on chromosome section 9q and CGH, gains are reported in childhood adrenocortical tumors by Figueiredo et al. [1999], and Dohna et al. [2000] concerning adults and children. On the other hand, Kjellman et al. [1996] did not find gains of chromosome 9q in adults in a series of 8 ACC.

Our results are slightly different to findings published by Almeida et al. [2010] who reported only 2 of a total of 27 adrenocortical carcinomas in adulthood presenting increased *SF1* gene copy number using multiplex ligation-dependent probe amplification (MLPA). The difference between Almeida et al. [2010] and our data may be rooted in different methods, i.e. MLPA *vs.* FISH, and thus, resulting from differing sensitivity. An highly speculative, alternative explanation could be that genic variations of the SF1 gene are present between adult ACC patients in Brazil and Europe.

As discussed earlier, all 58 evaluated normal and benign adrenocortical tumor tissues as well as 158 (98 %) ACC presented SF1 protein expression H-scores between 1 and 3 (see Table 5.2 on page 43). In the next step, we analyzed the correlation of SF1 H-score and *SF1* gene copy number in 23 ACC samples. The analysis revealed that 1 case (33 %) of the H-score 0 group (SF1 protein negative) had gains, 3 cases (30 %) of H-score 1 group presented gains,

one case (17 %) each a loss and amplification and 2 cases gains of H-score 2 group, and 1 case (25 %) of H-score 3 group had gains in *SF1* gene (see Figure 5.8 on page 53). Thus, the obtained results suggest that at least in a subset of samples one or several of the following mechanisms might be responsible for the discrepancy of the results on gene and mRNA level: *i.)* mRNA halflife of SF1 is increased (or increased translation is present), *ii.)* increased SF1 protein stability due to post-translational modifications, *iii.)* Ubiquitin-mediated degradation might be inhibited/decreased, or *iv.)* preferred expression of SF1 upstream targets occurs in adrenocortical carcinoma. Further support for one (or even more) of those hypotheses comes from the fact that both metastases presented normal *SF1* gene copy number but were characterized by H-score 1. Our data implies that the gene *SF1* by itself is not responsible for the tumorigenic role of SF1 protein in adult ACC.

It is important to note that our FISH study is not able to explain the difference in survival analysis between adrenocortical adenomas and carcinomas concerning SF1 protein. All adenomas presented normal gene copy number of *SF1* whereas adrenocortical carcinomas presented normal gene copies, gains, amplification and loss of *SF1* — although benign and malignant tumors presented strong SF1 protein staining in the majority of cases.

6.4 Is our cohort representative for ACC patients?

Our research was performed on the world-wide largest adrenocortical carcinoma cohort. Nevertheless, we have to verify that our cohort represents well published patient characteristics in this disease. The age distribution of adrenocortical carcinoma has 2 peaks, i.e. childhood and adulthood [Luton et al., 1990, Wajchenberg et al., 2000]. All patients in our study were recruited from the latter. Our data regarding age in adult ACC is in agreement with its usual occurence in the 4th–5th decade (see Table 5.1 on page 40). Sex distribution of primary ACC in the presented cohort was approximately 1.8 (48/85 [m/f]) which matches the distribution of 1.5 [Luton et al., 1990, Wooten and King, 1993, Icard et al., 2001, Koschker et al., 2006] (see Table 5.1). Average tumor size of investigated ACC was 10.8 cm which is between 10 cm and 12 cm as stated by Kebebew et al. [2006] and Ng and Libertino [2003] (see Table 5.1 on page 40). Furthermore, the classification of the cases according to the different tumor stages of the ENSAT system [Fassnacht et al., 2009] reflects quite well the diagnose of the majority of patients in stage II and III (see Table 5.1). Thus, our adrenocortical carcinoma cohort shows all the characteristics known from published series in the literature.

6.5 Limitations and strengths of this study

The study has several limitations, but also considerable strengths.

First, the sample size of 161 ACC and 73 non-adrenal tumor samples is small in comparison to other tumor entities. But considering that ACC is a rare carcinoma (1–2 cases per year and million) our patient cohort is until now the world-wide largest for immunohistological analyses.

Second, data were collected in a retrospective manner and therefore an unknown bias can not be excluded.

Third, the sample size of FATE1 and DAX1 immunohistochemistry, and *SF1* FISH investigation is particularly small. This can explain the low values concerning sensitivity, negative and positive predictive value in the immunohistochemical investigations. Furthermore, small

sample sizes prevent an encompassing view about the protein of interest, or, in the case of *SF1* FISH, gene copy number (i.e. only 1 metastases, 2 local recurrences, and 6 adenomas). Especially, tissue loss during experimental work is a problem when dealing with rare tumors and limited amount of tumor material. Thus, it was not possible to ensure encompassing availability of those tissues. Notably, only a very few studies have investigated more than 50 ACC samples. Therefore, our study cohort is still remarkably large.

Fourth, we combined H-scores which should ideally be treated as single groups. For example, we combined SF1 and FATE1 protein negative samples with the low SF1 and FATE1 protein expression group, respectively. The grouping was neccessary to ensure sufficient sample sizes aiming at almost equal distribution for evaluation of patient survival for each protein staining group.

Fifth, all survival analyses were performed *excluding* patients hormonal status in the ACC cohort to ensure sufficient sample size. Therefore, we cannot judge the influence of hormone secretion in the context of our markers.

Sixth, our study did not address in FISH investigation if gains (or loss/amplification) encompassed the full length gene. Therefore, it might be possible that (some) gainswere caused by an increased number of given gene sections of *SF1* — e.g. exon 2 is present more than once per single gene.

Seventh, due to the heterogenous presentation of ACC, it can be difficult to differentiate it from other retroperitoneal tumors. To guarantee diagnosis of adrenocortical carcinoma, the German reference pathologist Prof. Saeger (Hamburg) has reviewed the slide of all samples and confirmed the diagnosis in all reported patients.

6.6 Future prospects

Investigation of the pathologically-relevant role of SF1 in patients with adrenocortical carcinoma was crowned with success. But to further improve the diagnostic value of SF1 immunohistochemistry, other markers for differentiation of benign and malignant adrenal tumors have to be identified. Possible candidates are other SF1 interaction partners. *SF1* expression correlates with Gata-6 which is linked to the benign phenotype of adrenocortical tumors [Kiiveri et al., 2005]. Further possible targets could be nuclear proteins SOX15, TEAD4, CENP and TAF5-like RNA polymerase which are shown to activate SF1 [Sakai et al., 2008]. FATE1 and DAX1 are no ideal candidates, as shown in this study. Other factors like CITED2 and PBX1, which were shown to be also activated by SF1 in a dosage-dependent manner and to be involved in adrenogenesis [Ferraz-de Souza et al., 2009], could be examined. In a small series, it has been shown that, for instance, CITED2 protein is expressed in ACC [Haase et al., 2007]. Further promising candidates might be WNT4 and β-catenin. It was published that *WNT4* overexpression inhibits synergy of SF1 and β-catenin [Jordan et al., 2003] and that *WNT4* mRNA level is higher in Conn's adenoma than in Cushing's adenoma. It is worth to be noted that mutations of *β-catenin* are frequent in benign and malignant adrenocortical tumors and that somatic activation mutations of *β-catenin* constitutively activated T cell factor-dependent transcription [Tissier et al., 2005]. Furthermore, isoform T cell factor 4N interacts with β-catenin potentiating its interaction with SF1 [Kennell et al., 2003]. Considering this background, the report of Berthon et al. [2010] revealing constitutive *β-catenin* activation as driving adrenal cancer development may been seen in different light.

We showed that expression of *SF1* correlates significantly with overall and recurrence-free survival in patients presenting adrenocortical carcinoma but not adenoma. Evaluation of the gene copy number of *SF1* cannot explain the difference in survival since adrenocortical adenomas presented normal gene copy numbers — as the majority of adrenocortical carcinomas.

Little is known concerning FATE1 — at least in human adrenocortical tumors. It is recommendable to use it and SF1 as combined markers for differentiation of adrenocortical tumors, although it was shown previously that SF1 drives expression of *FATE1 in vitro* [Doghman et al., 2007]. Considering our large sample size of adrenal tumors and FATE1-specific results, there might be a value of FATE1 in immunotherapy. Nontheless, FATE1 and the combinations SF1/FATE1, and SF1/FATE1/DAX1 might be used for prognosis concerning overall survival. Furthermore, SF1/FATE1 is a good tool to investigate recurrence-free survival. But one has to consider that staining with additional antibody is time, money, and tissue consuming and that SF1 alone is a well suited prognostic marker.

Vilain et al. [1997] showed that *DAX1* is upregulated by binding of SF1 protein to its responsive element in the *DAX1* promoter. Although our observed results are aquired by immunohistochemistry they support the hypothesis of Vilain et al. [1997] — in adrenocortical tumors — since a higher ratio of DAX1-positivity was observed, although almost all distant metastases were negative for DAX1.

Since DAX1 is proposed to be involved in mouse embryonic stem cell self-renewal and pluripotency [Loh et al., 2006, Wang et al., 2006, Kim et al., 2008] and its knock-down is associated with differentiation of embryonic stem cells [Khalfallah et al., 2009] our findings might support the hypothesis that adrenocortical cancer cell context resembles fetal adrenal gland cell context where stem-cell like cells are proposed to be involved in organogenesis (see Mesiano and Jaffe [1997] for a review). Of note, in human embryonic stem cells, *DAX1* expression is low and inconsistent [Xie et al., 2009]. Interestingly, DAX1 is reported in human fetal adrenal glands with staining throughout definitive and fetal zone decreasing to the inner fetal zone [Battista et al., 2005].

Up to now, treatment options for ACC are very limited. Since SF1 protein is expressed by almost all adrenocortical tumors, it might be valuable as target for primed immune cells. Further alternatives could be therapies that inhibit its activity using inverse agonists. Considering this approach seems promising [Del Tredici et al., 2008, Doghman et al., 2009]. Inhibitors of histone deacetylases might also be valuable tools. Chen et al. [2007] showed that a few inhibitors are capable to increase ubiquitinylation followed by degradiation of SF1. Another strategy could be the development and directed transport of hammerhead ribozymes (catalytical active RNA molecules) to target *SF1* transcripts. This method was shown to be effective in cell lines [Materna et al., 2005].

6.7 Key messages

- SF1
  - SF1 is the best marker for differentiation of adrenocortical tumors from other retroperitoneal tumors.
  - SF1 does not discriminate benign from malignant adrenocortical tumors.
  - SF1 is an excellent prognostic marker for ACC patients' overall and recurrence-free survival.

  - There is no correlation between SF1 protein expression and its gene copy number suggesting post transcriptional regulation.

- FATE1
  - *FATE1* expression is not helpful for differentiation of adrenocortical tumors from other retroperitoneal tumors.
  - FATE1 is also no valuable diagnostic tool for differentiation of benign and malignant adrenocortical tumors.
  - FATE1 is only of some prognostic value for overall survival.
  - If the results of the immunohistochemistry of SF1 and FATE1 are combined, these combined factors are slightly better prognostic parameters than SF1 alone.

- DAX1
  - *DAX1* expression alone and in combination with SF1 and/or FATE1 has no diagnostic and prognostic value in adrenocortical tumors.

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Appendices

A
Abbrevations

For nucleic acids and its building blocks as well as amino acids one and three letter abbrevations according to the White Book of IUPAC-IUBMB Joint Commission on Biochemical Nomenclature and Nomenclature Commission of IUBMB (`http://www.chem.qmul.ac.uk/iupac/bibliog/white.html`) were used. These conventions were also applied for physical units, units of measurement and possible prefixes. Nomenclature of nuclear recepetors is performed as suggested by NRNC [1999], Escriva et al. [2000] and Thornton and DeSalle [2000]

⌀	Diameter
%	Percent
Å	Angstrom
°C	Degree celsius
μ	Micro-
aa	Amino acid
ACC	Adrenocortical carcinoma
ACT	Adrenocortical tumor
ACTH	Adrenocorticotrophic hormone
Ad4BP	*Adrenal 4 binding protein*
bp	Basepair
BSA	Bovine serum albumin
ca.	*circa*
CITED2	*Cbp/p300-interacting transactivator, with Glu/Asp-rich carboxy-terminal domain, 2*
C-terminal	Carboxy terminal
CT43	*Cancer/testis antigen 43*
d	Coat thickness
Da	Dalton
DAX1	*Dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X, gene 1*
DHEA(S)	Dehydroepiandrosterone (sulfate)
DNA	Deoxyribonucleic acid
dNTP	Desoxyribonucleosid triphosphate
ds	Double stranded
E	Extinction
E. coli	*Escherichia coli*
EDTA	Ethylenediaminetetraacetic acid

e.g.	*Example gratia*
ERK1/2	*Extracellular signal-regulated kinases 1/2*
et al.	*Et alii*
etc.	*Et cetera*
EtOH	Ethanol
FATE1	*Fetal and adult testis expressed 1*
FFPE	Formalin-fixed, paraffin-embedded tissue
Fig.	Figure
FISH	Fluorescence *in situ* hybridization
g	Gramm
h	Hour
HRP	Horseradish peroxidase
i.e.	*Id est*
IGF-II	*Insulin-like growth factor II*
IHC	Immunohistochemistry
k	Kilo-
kbp	Kilo basepair
l	Litre
m	Milli-
M	mol/l, molar
MeOH	Methanol
min.	Minute
ml	Milliliter
M_r	Relative molecular mass
mRNA	Messenger RNA
MSH	Melanocyte-stimulating hormone
MW	Molecular weight
n	Nano-
nm	Nanometer
N-terminal	Amino terminal
No.	Number
NR0B1	*Nuclear receptor subfamily 0, group B, member 1*
NR5A1	*Nuclear receptor subfamily 5, group A, member 1*
OD_{230}	Optical density at 230 nm
OD_{260}	Optical density at 260 nm
OD_{280}	Optical density at 280 nm
OD_{320}	Optical density at 320 nm
p	Pico-
p.A.	*Per analysi*
PBS	Phosphate buffered saline
PBX1	*Pre-B-cell leukemia homeobox 1*
pH	*Pondus Hydrogenii*, *potentia Hydrogenii*, negative common logarithm of oxonium ion concentration
POMC	*Proopiomelanocortin*
R0	Complete tumor resection
R1	Propably incomplete tumor resection
RX	Resection border of the tumor is not clearly assessable

RNA	Ribonucleic acid
rpm.	Rounds per minute
RT	Room temperature
SF1	*Steroidogenic factor 1*
SSC	Saline sodium citrate
STAT3	*Signal transducer and activator of transcription 3*
ss	Single stranded
Tab.	Table
TE	Tris-EDTA
Tris	Tris-(hydroxymethyl)-aminomethane
TMA	Tissue microarray
TN	Tris-HCl, NaCl
TNB	Tris-HCl, NaCl, blocking reagent
TNT	Tris-HCl, NaCl, Tween 20
U	Unit (enzyme unit)
UV	Ultraviolet
V	Volt
(v/v)	Percent by volumen
Vol.	Volumen
W	Watt
(w/v)	Percent by weight

One and three letter abbrevations of amino acids

A, Ala	Alanine
B, Asx	Asparagine or aspartic acid
C, Cys	Cysteine
D, Asp	Aspartic acid
E, Glu	Glutamic acid
F, Phe	Phenylalanine
G, Gly	Glycine
H, His	Histidine
I, Ile	Isoleucine
K, Lys	Lysine
L, Leu	Leucine
M, Met	Methionine
N, Asn	Asparagine
P, Pro	Proline
Q, Gln	Glutamine
R, Arg	Arginine
S, Ser	Serine
T, Thr	Threonine
V, Val	Valine
W, Trp	Tryptophan
Y, Tyr	Tyrosine
Z, Glx	Glutamine or glutamic acid

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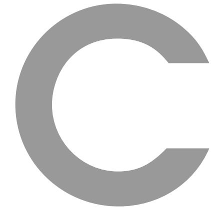

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