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Suppression of Cytochrome P450 4B1: An Early Event in Adrenocortical Tumorigenesis

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Abstract

Background—Adrenocortical carcinoma (ACC) is a rare neoplasm with a poor prognosis. Conversely, adrenocortical adenomas (ACAs) are common and benign. Despite their shared histological origin, little evidence exists that ACA arises from ACC. Recent genetic analyses of ACC have shown recurrent gene copy deletion of *CYP4B1*, a cytochrome P450 isozyme. This study investigates a potential role for CYP4B1 in modulating adrenocortical tumorigenesis and/or conferring chemoresistance to ACCs.

Methods—Using TaqMan real-time qPCR techniques, we investigated *CYP4B1* expression in normal adrenal cortex ($n=10$), histologically confirmed ACAs ($n=10$), and ACCs ($n=10$). ACC cell lines were enforced to express *CYP4B1* and effects on cell death and enhanced mitotane and cisplatin sensitivity were tested.

Results—Gene expression analyses demonstrated suppression of *CYP4B1* in 100% of ACAs (10/10) and ACCs (10/10) tested. Average relative expression of *CYP4B1* was reduced at 0.19 (0.01–0.50; $P < .01$) in ACAs, and nearly absent in ACCs (0.01; 0.00–0.05; $P < .01$). Protein expression correlated with mRNA expression. Ectopic expression of *CYP4B1* promoted cytotoxicity and increased chemosensitivity in ACC cell lines.

Conclusions—*CYP4B1* is silenced in adrenocortical tumors (ACTs) and may contribute to tumorigenesis and chemoresistance. Sensitization of ACC cells engineered to overexpress *CYP4B1* further supports this notion.

Background

Adrenocortical carcinoma (ACC) is a rare endocrine malignancy that portends a poor prognosis. It occurs in 0.5 – 2 people per million per year and has a 5-year survival rate of only 16–38%.^{1–4} The majority of these tumors (65–85%) will exhibit hormonal hypersecretion of cortisol, aldosterone, or sex steroids but can be nonfunctional as well.⁵ Due in part to its rarity, the underlying molecular and genetic pathogenicity of ACC is poorly understood. The lack of reliable genetic markers undermines therapeutic decision-making and causes significant problems in diagnosis, prognosis, and management. Inherited

disorders such as Beckwith-Wiedemann syndrome and Li-Fraumeni are found in a subset of patients, however, the majority of cases are sporadic.^{2,6} Investigators have thus far focused on rare genetic mutations in known oncogenes such as *TP53*, *IGF2*, and *APC*, however, the benefits of this research has yet to reach patients.^{1,7}

While increased access to high-throughput sequencing has facilitated exploration of the genetic landscape of ACC, complete R0 surgical resection remains the only prospect for a durable cure.^{5,8,9} Advancements in treatment have been limited due to the inherent chemoresistance and lack of knowledge of the molecular drivers of ACC. Mitotane (1,1-dichloro-2-(o-chlorophenyl)-2-(p-chlorophenyl) ethane), the classical first-line agent in the treatment of ACC, acts by directly lysing adrenal cortical cells and can decrease hormone hypersecretion. Recent data has emerged that suggests combination therapy with cisplatin may increase response rates and progression-free survival.^{10,11}

Altered cellular metabolism is a prominent feature of ACC and is an important area for oncologic research.⁷ Essential elements in this system are the cytochrome P450 (CYP) enzymes. This superfamily consists of more than 50 heme-thiolate proteins that play essential roles in both the biosynthetic and catabolic pathways of vitamins, fatty acids, prostaglandins, bile acids, and steroids. Steroid production can strongly influence cancer growth and impact cellular metabolism, especially in endocrine organs like the adrenal gland.¹²

Because CYP enzymes constitute one of the most important mechanisms for human drug metabolism, they are of central importance in chemoresistance.^{13,14} Altered CYP expression has been shown to increase rates of anti-cancer drug degradation, decreasing the intended cytotoxic effect.^{13,14} Additionally, while not considered typical oncogenes or tumor suppressors, differential expression of cytochrome P450 genes has been demonstrated to be associated with pancreatic, breast, bladder, and lung cancer.^{15–18}

Recent bioinformatic analysis of copy number variations (CNVs) in ACC displayed widespread genomic instability and multiple arm-level and focal alterations in the cytochrome P450 genes.¹⁹ Among the CYP genes, cytochrome P450 4B1 (*CYP4B1*) was found to be deleted in numerous samples. Because of the crucial functions of CYP enzymes in steroid metabolism and adrenal gland physiology, we investigated the potential role of *CYP4B1* in adrenocortical tumors (ACTs).

CYP4B1 is unique among the cytochrome P450 class in that it is involved in both endobiotic and xenobiotic metabolism. It selectively hydroxylates fatty acids and may exert toxicological effects via activation of aromatic amines whose byproducts can be cytotoxic under specific conditions.²⁰ Research on bladder cancer has demonstrated that the absence of wild type *CYP4B1* is associated with tumor carcinogenesis.¹⁸ Additionally, *CYP4B1* has differential expression patterns in lung cancer compared to normal tissue.¹⁶ The association of *CYP4B1* with other malignancies and the demonstrated copy number loss in ACC led us to investigate its potential role in adrenocortical tumors.

Methods

Patients and tissue collection

Patients included in this cohort were treated at Yale New-Haven Hospital from August 2003 to July 2015. ACC ($n = 10$), ACA ($n = 10$), and adjacent normal tissue samples ($n = 10$) were collected by Yale Pathology Tissue Service intraoperatively. ACCs were sequentially collected from 2003 onward, while ACAs were selected based on the time since operation. All the patients in the ACA cohort had their operations between 2012 and 2014. Adrenal tissue samples were examined by an experienced endocrine pathologist for histological identification. Additional formalin-fixed paraffin-embedded samples from the same patients were used for immunohistochemistry. Written informed consent was obtained from all patients involved. Protected health information was acquired and maintained in accordance with regulations as specified by the Health Insurance Portability and Accountability Act (HIPAA). The consent waiver and all procedural aspects of this study were approved by the Yale University Institutional Review Board.

Gene Expression Analysis

RNA was isolated from fresh frozen samples using the RNeasy Plus Mini Kit (Qiagen, Hilden, Germany). Quantity and quality of isolated RNA was assessed by spectrophotometry (NanoDrop Technologies). Two hundred ng of RNA was used for cDNA synthesis with the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA). Quantitative RT-PCR was performed on a CFX96 Real-Time System thermocycler (Bio-Rad) using TaqMan PCR master mix with primers and probes specific to CYP4B1 (all from Applied Biosystems, Foster City, CA). Relative expression between samples was normalized to endogenous ribosomal protein, large, P0 (*RPLP0*) expression levels. Two independent experiments were performed with duplicate samples for all assays. Relative expression levels were calculated using the Livak method.²¹

Immunohistochemical analysis

Five μm thick sections of histologically confirmed ACC, ACA, and normal adrenal tissue from formalin fixed paraffin embedded (FFPE) tissue samples were collected for study. Two representative samples of each tissue type were selected for immunostaining. Using standard immunohistochemistry protocols, target epitopes were detected using rabbit anti-*CYP4B1* polyclonal antibody (Santa Cruz, Dallas, TX) followed by anti-rabbit HRP conjugated monoclonal secondary antibody (Santa Cruz).²² DAB (3,3'-diaminobenzidine tetrahydrochloride) was utilized for antigen detection (Life Technologies, Carlsbad, CA). Sections were counterstained with hematoxylin and mounted using immunohistomount (Santa Cruz).

Ectopic expression of CYP4B1 in ACC cell lines

Standard cell culture experiments were performed as previously described.²² The human ACC cell lines SW-13 and NCI-H295R were purchased from the American Type Culture Collection (Manassas, VA). SW-13 cells were maintained under sterile conditions in Dulbecco's Modified Eagle's medium (DMEM) supplemented with 10% certified fetal

bovine serum and 10,000 U/mL penicillin/streptomycin (Life Technologies) in a standard humidified incubator at 37.0 °C and 5% CO₂. NCI-H295R cells were grown in DMEM/F-12 containing 5% NuSerum, 0.1% ITS premix, and 10,000 U/mL penicillin/streptomycin (Life Technologies). Myc-DDK tagged pCMV6-Entry, pCMV6-Entry/GFP, and pCMV6-Entry/*CYP4B1* plasmid vectors (Origene, Rockville, MD) were used to transfect cells that were grown to 70–80% confluence.

Transient transfection was performed in 24-well plates using Lipofectamine 2000 according to the manufacturer's recommendations (Life Technologies). Transfection efficiencies were determined by quantifying the percentage of GFP-expressing cells from parallel transfections carried out using pCMV6-Entry/GFP vectors. 24 hours after transfection, cells were treated with chemotherapy agents or the appropriate diluents. Mitotane and cisplatin were dissolved in 100% ethanol or distilled water respectively (Spectrum Chemical, New Brunswick, NJ). Cells were treated with either (1) mock, (2) mitotane 5 μM, (3) mitotane 50 μM, (4) mitotane 50 μM plus cisplatin 700 nM, or (5) untreated. After 24 hours of treatment, cell viability was measured using Trypan blue assay.²² A minimum of two independent experiments were performed with duplicate samples in each assay. Standard Student's *t* test was used to assess statistical significance in normally distributed data sets. A *P* value < .01 was considered significant.

Results

Patient Characteristics

Both the ACA and ACC groups had a predominance of female patients with 60% and 80% respectively. Average age at the time of operation was 55.5 ± 10.4 for ACC and 45.7 ± 14.0 in ACA. Mean tumor diameter was nearly 9 cm greater in ACC than ACA (ACC = 11.3 cm [6.0 – 14.0]; ACA = 2.5 cm [1.1 – 6.1]). The majority of tumors had biochemically unequivocal hormone hypersecretion. ACCs were more often cortisol producing (4/10), while 50% of ACAs secreted aldosterone (5/10; Table I).

Reduced Expression of *CYP4B1*

Gene expression analyses demonstrated suppression of *CYP4B1* in 100% of ACA (10/10) and ACC (10/10) samples when compared with adjacent histologically normal adrenal tissue (Fig 1, 2 and 3). Overall, there was a correlation between tumor dedifferentiation and decreased *CYP4B1* expression. Average relative expression of *CYP4B1* in ACA was 0.19 (0.01 – 0.50) and was significantly reduced when compared to expression levels in the normal adrenal cortex (*P* < .01; Fig 1, 2). Expression was nearly absent in ACC samples and significantly less than ACA and normal (Average = 0.01; 0.00 – 0.05; *P* < .01; Fig 1, 3).

Immunohistochemical detection of protein expression

Histologically confirmed ACA, ACC, and normal adrenal samples were chosen from among the cohort to evaluate *CYP4B1* protein expression. In normal adrenal tissue, immunostaining in the cytoplasmic and perinuclear regions was observed. Overall protein levels in ACA were markedly decreased compared to normal and undetectable in ACC (Fig 4).

Enforced expression of CYP4B1 in ACC cells

Stable transfection of *CYP4B1* in NCI-H295R cells resulted in immediate loss of viability. Stable neomycin-resistant SW-13 cells selected also eventually lost the ability to express *CYP4B1*. Since the enforced stable expression of *CYP4B1* appeared to be toxic to both ACC cell lines, we performed transient expression of *CYP4B1* in relatively more tolerant SW-13 cells to determine if the presence of the protein was initiating cytotoxicity. Cells were also tested to determine whether standard ACC chemotherapy agents accentuate CYP4B1-promoted cytotoxicity. The effects of the medications tested were similar to those that have been reported before (Fig 5, 6).²³ As could be predicted from the outcome of stable expression, expression of *CYP4B1* alone was found to be sufficient to promote loss of ACC cell viability (Fig 5). This pattern of *CYP4B1* promoted cellular toxicity persisted independent of the chemotherapy regimen tested (Fig 5). While control cells treated with low-dose mitotane therapy (5 μ M) did not result in cytotoxicity, cells transfected to express *CYP4B1* had significantly decreased viability and increased cell death ($P < .01$; Fig 5, 6). Addition of cisplatin to mitotane resulted in increased loss of viability compared to mitotane (50 μ M) alone, and was further accentuated by expression of *CYP4B1* (Fig 5). These experiments suggest a role for CYP4B1 in sensitizing dedifferentiated adrenocortical cells to cell death.

Conclusions

While the molecular etiology of adrenocortical carcinogenesis has yet to be fully characterized, substantive advances have been made in understanding the disease. However, cataloging the genetic and epigenetic changes that initiate and drive ACC has been stifled by both its rarity and inherent genetic complexity. Specifically lacking are studies that explore the effects of genetic change in the context of the complex metabolic milieu of the adrenal cortex. This study focuses on the potential role of a seminal member of the CYP family in the initiation and/or progression of ACCs.

In this study, we demonstrate significantly reduced expression of *CYP4B1* in 100% of adrenocortical tumors, a previously undescribed event of potential metabolic and carcinogenic significance. Although the mechanism underlying observed sequential suppression of *CYP4B1* in ACTs has not been completely clarified, copy number loss appears to contribute to the silencing of *CYP4B1*. Alternatively, epigenetic alteration may be a more dominant influence as the global silencing observed in all ACTs studied would not be explained by the lesser degree of gene copy loss.²⁴ Moreover, the downregulation appears to follow a graded trend along with the dedifferentiation of the adrenal cortex (Fig 4).

CYP4B1 dysregulation has been noted in numerous malignancies. Unlike the present study, bladder and lung tumors show upregulation, not suppression, of *CYP4B1*.^{16,18,25} However, in bladder cancer, this shift appears to be associated only with allelic variants and not observed in the wild type protein.¹⁸ Repressed activity of CYP4B1 through missense mutations and deletions may allow malignant transformation. It is very likely that the metabolically intense endocrine physiology of the adrenal gland requires CYP4B1 for steroid synthesis, which, when perturbed, may lead to metabolic chaos and cell death. Since a specific role for CYP4B1 in the normal adrenal cortex is not yet known, it is difficult to

speculate on the impact of its dysregulation or the possible effects of its silencing on adrenocortical dedifferentiation or carcinogenesis.

Of special interest, is the observation that both transient and stable expression of *CYP4B1* resulted in toxicity in two well-studied ACC cell lines. Although the effect needs to be confirmed using inducible promoters, the preliminary results point towards a tumor suppressor role for *CYP4B1* in ACC. The mechanism by which an enzyme uncommonly expressed in other tissues maintains a gatekeeper function in the context of the adrenal cortex is of immense interest, and needs to be studied further.²⁶

Results of our experiments assessing the cytotoxic effect of *CYP4B1* further support its role as a cell-death promoter in ACTs. Treatment with both mitotane and cisplatin in *CYP4B1*-expressing cells decreased viability in the two ACC cell lines studied. In the doses and combinations tested, presence of *CYP4B1* appears to accentuate toxicity irrespective of the agent used. It is also noteworthy that *CYP4B1* promoted significant cytotoxicity in SW-13 cells with low-dose mitotane (5 μ M; Fig 5, 6). This apparent chemosensitization is clinically important as it may confer increased efficacy to anti-cancer drugs. Raising concentrations of peritumoral chemotherapy effectively shifts the dose-response curve, potentially reducing the risk of systemic toxicity.

Despite their common histological origin and shared profile of hormone secretion, there is little evidence that ACC arises from ACA. The graded decrease in expression of *CYP4B1* and the observed correlation with adrenal dedifferentiation suggest at least a physiologic parallel between these distinct neoplasms. However, the small cohort size and the lack of further detailed examination prevent this study from making any concrete connection between these entities. In summary, this study offers new insight into the potential roles of *CYP4B1* in the overall process of adrenal tumorigenesis and ACC chemoresistance.

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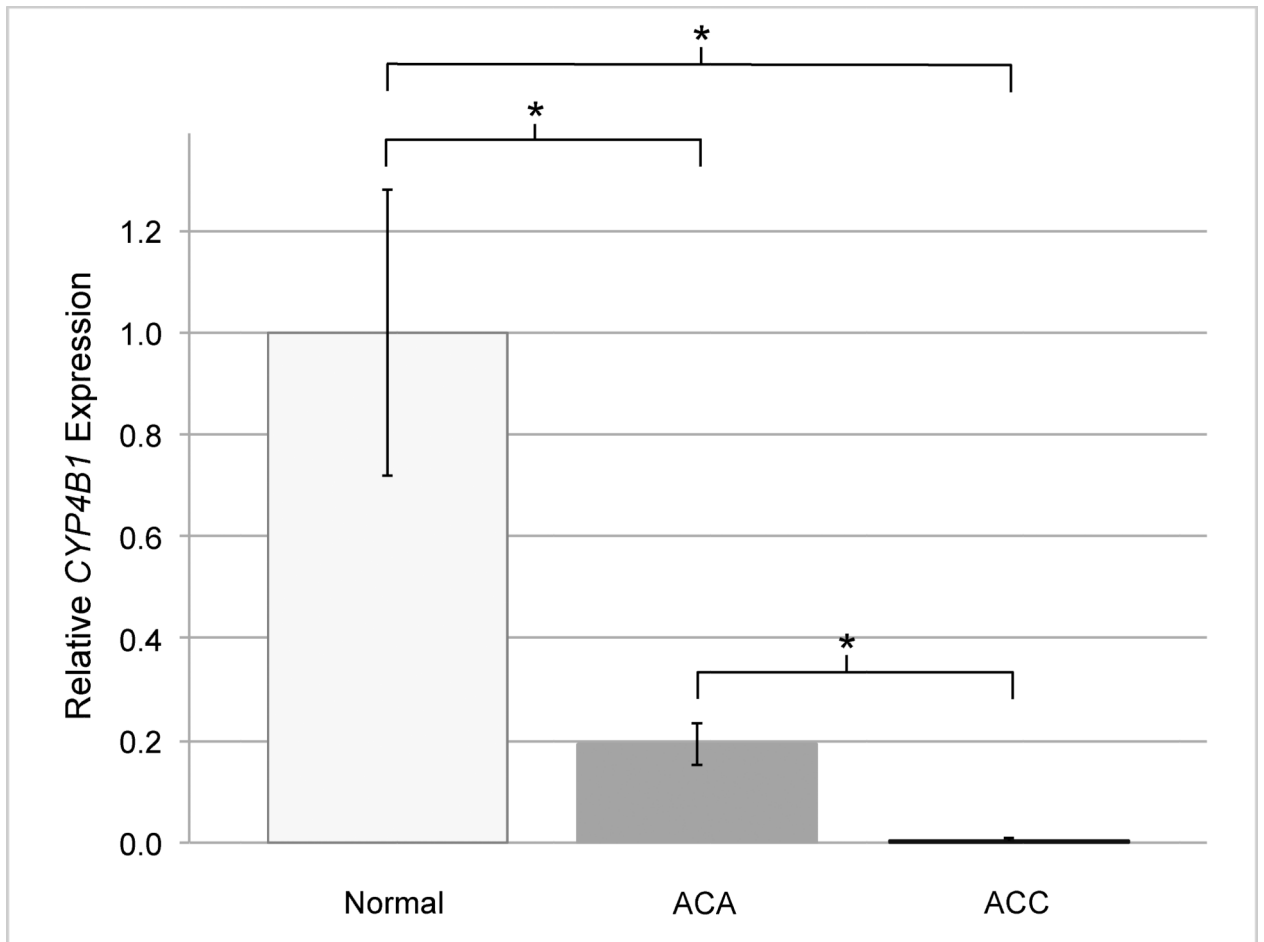


Fig 1. Average relative expression of *CYP4B1* is significantly decreased in adrenocortical tumors relative to normal adrenal tissue. Mean mRNA expression in normal samples ($n = 10$) is normalized to 1 and relative expression in ACAs ($n = 10$) and ACCs ($n = 10$) are shown. Statistical significance was calculated using 2-tailed t test and P value $< .01$ was considered significant. Error bars, SEM. (* = P value $< .01$)

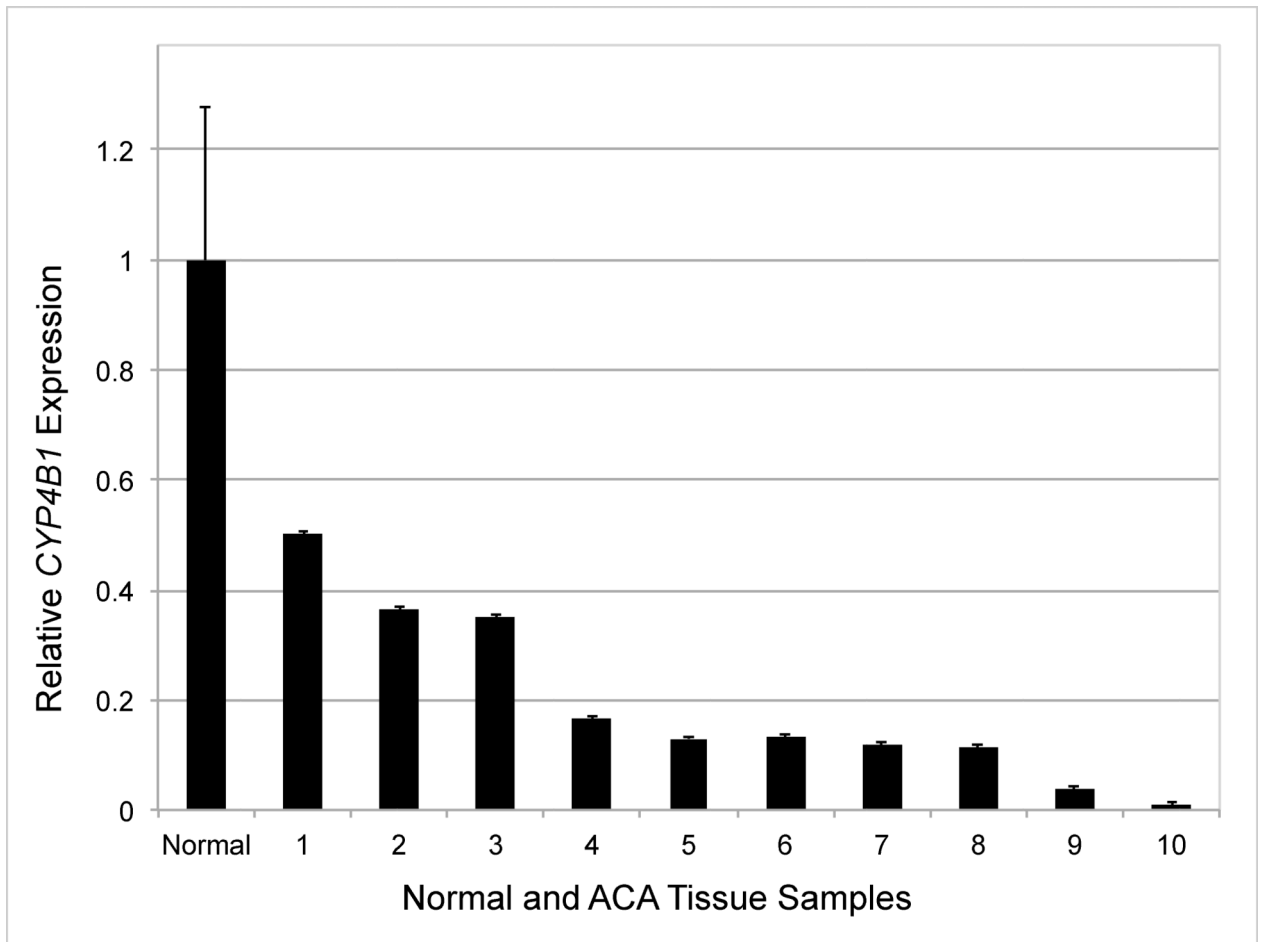


Fig 2. *CYP4B1* expression in individual adrenocortical adenoma (ACA) samples. Mean mRNA expression in normal samples ($n = 10$) is normalized to 1 and relative expressions in individual ACAs ($n = 10$) are shown. X axis: Normal and ACA tissue samples (numbered 1–10). *Error bars*, SEM.

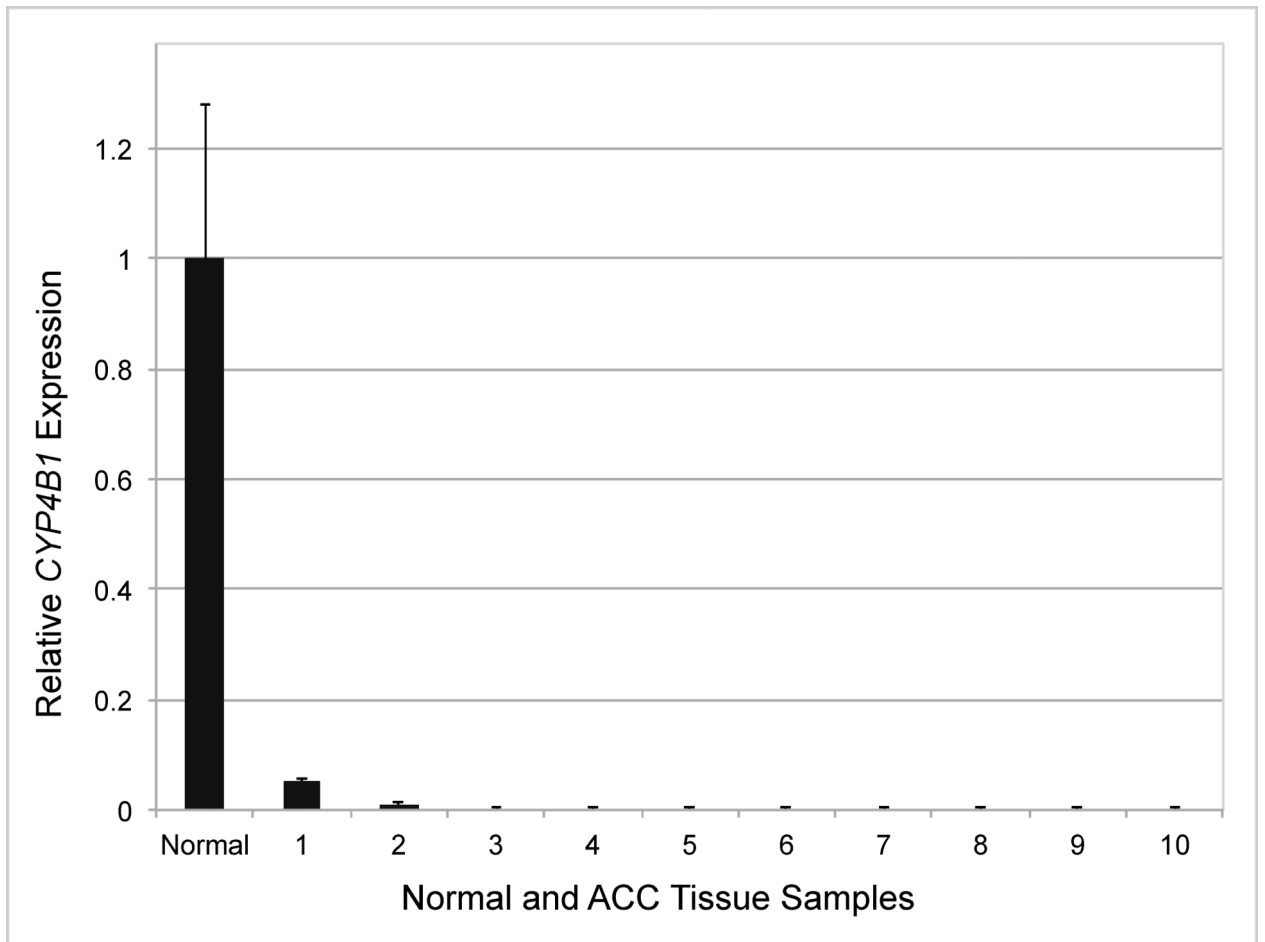


Fig 3. *CYP4B1* expression in individual adrenocortical carcinoma (ACC) samples. Mean mRNA expression in normal samples ($n = 10$) is normalized to 1 and relative expressions in ACCs ($n = 10$) are shown. X axis: Normal and ACA tissue samples (numbered 1–10). Error bars, SEM.

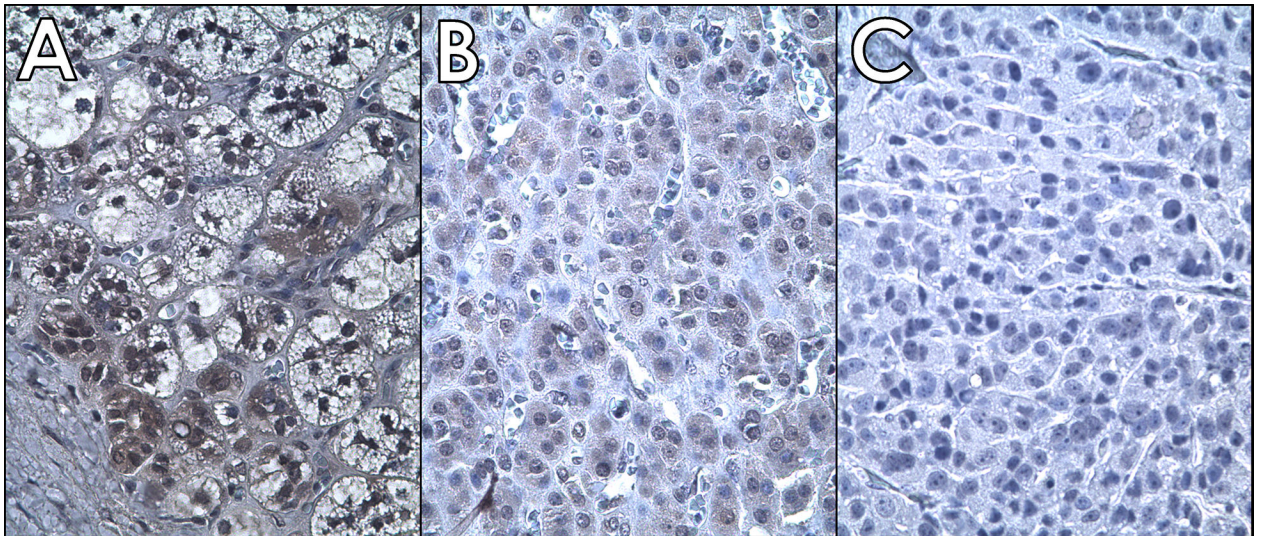


Fig 4. Immunohistochemical analysis confirmed reduced expression of CYP4B1 in Adrenocortical Tumors (A: Normal adrenal cortex, B: Adrenocortical adenoma, C: Adrenocortical carcinoma). Original magnification - 400X. Brown staining indicates CYP4B1 protein and dark blue indicates nuclear staining by hematoxylin.

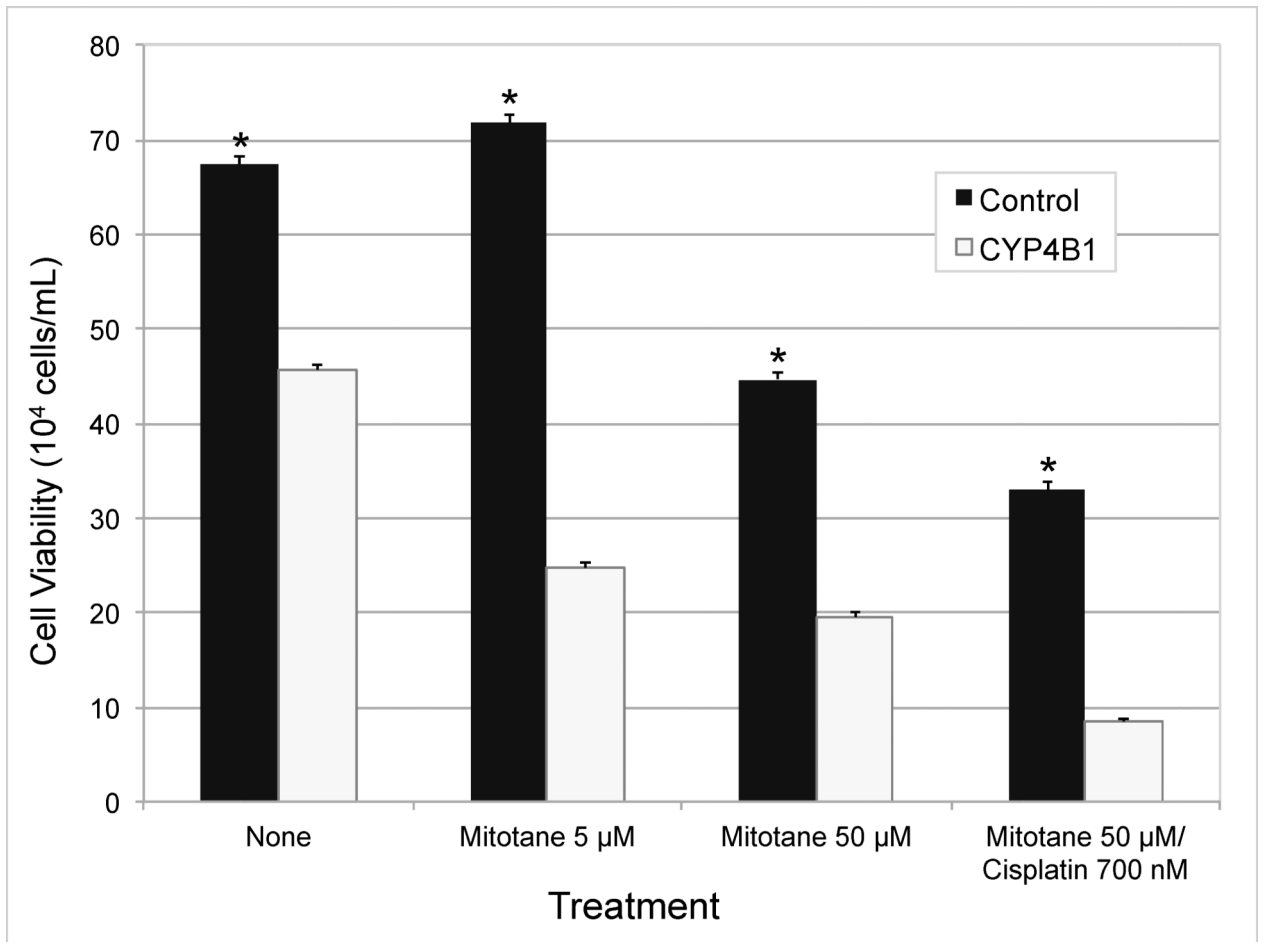


Fig 5. Transient expression of *CYP4B1* in ACC cell line (SW-13) decreases viability and sensitizes cells to chemotherapeutic agents. Twenty-four hours post-transfection, cells were treated with or without chemotherapy. Twenty-four hours after, cell viability was measured via Trypan blue assay. Statistical significance was calculated using 2-tailed *t* test and *P* value < .01 was considered significant. (* = *P* value < .01)

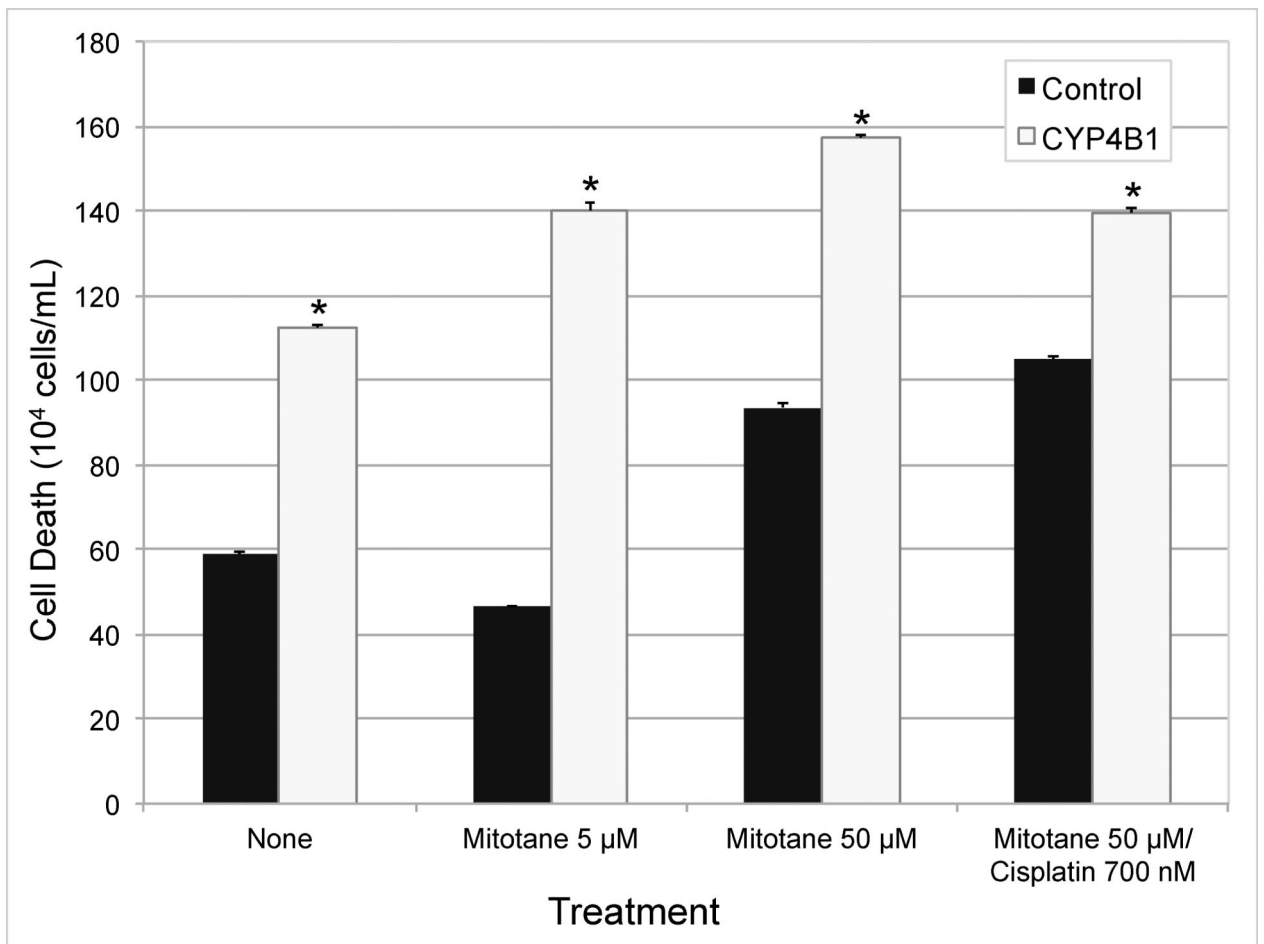


Fig 6. Transient transfection of ACC cell line (SW-13) with *CYP4B1* vector increases cell death and sensitizes cells to chemotherapeutic agents. Twenty-four hours post-transfection, cells were treated with or without chemotherapy. Twenty-four hours after, cell death was measured via Trypan blue assay. Statistical significance was calculated using 2-tailed *t* test and *P* value < .01 was considered significant. (* = *P* value < .01)

Table I.

Clinical features of patients with adrenocortical carcinoma (ACC) and adrenocortical adenoma (ACA)

Variable	ACC	ACA
Total (n)	10	10
Age \pm SD, y	55.5 \pm 10.4	45.7 \pm 14.0
Male	2	4
Female	8	6
Stage*		
I	0	n/a
II	2	n/a
III	5	n/a
IV	3	n/a
Tumor diameter, cm, mean (range)	11.3 (6.0 – 14.0)	2.5 (1.1 – 6.1)
Hormonal status		
Cortisol	4	2
Nonhyperfunctional	3	2
Estradiol	1	0
Multiple	1	1
Unknown	1	0
Aldosterone	0	5

* Staging criteria per American Joint Committee on Cancer (AJCC), 7th edition