Functional Significance of Cytochrome P450 1B1 in Endometrial Carcinogenesis

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Abstract
Cytochrome P450 1B1 (CYP1B1) catalyzes estrogen hydroxylation and activation of potential carcinogens. Here we explored the role of CYP1B1 in endometrial carcinogenesis. Immunohistochemical staining of endometrial carcinomas showed that CYP1B1 is up-regulated in endometrial cancers. To understand the functional significance of CYP1B1 up-regulation in endometrial cancers with regard to tumorigenesis, we used small interfering RNA-mediated approach to knockdown CYP1B1 in endometrial carcinoma cell line followed by functional assays. Further, to understand the molecular basis of the role of CYP1B1 in endometrial carcinomas, we profiled the expression of key pathway-specific genes and identified several components of cell cycle, apoptosis, and cell adhesion pathways that are potentially regulated by CYP1B1. CYP1B1 depletion in endometrial carcinoma cells leads to decreased cellular proliferation and induces G0-G1 cell cycle arrest. Significantly, CYP1B1 knockdown leads to down-regulated expression of cyclin E1, S-phase kinase-associated protein 2 (SKP2), minichromosome maintenance complex component 4 (MCM4), and RAD51 and up-regulation of p27kip1. Also, we identified cyclin E-binding protein (CEBP1) as a novel CYP1B1 target. Attenuation of CYP1B1 expression in endometrial carcinoma cells induces apoptosis and increases expression of IFN-β (IFN3), granzyme A (GRZA), and tumor necrosis factor-related apoptosis-inducing ligand (TRAIL). Importantly, CYP1B1 depletion decreased the invasive potential of the endometrial cancer cells and expression of melanoma cell adhesion molecule (MCAM). In conclusion, our data suggest that CYP1B1 up-regulation plays a crucial role in endometrial carcinogenesis by targeting multiple pathways. We speculate that CYP1B1 inhibition in endometrial carcinomas could be a useful therapeutic approach as it regulates several potential anticancer targets like cyclin E1, Skp2, and TRAIL. [Cancer Res 2009;69(17):7038–45]

Introduction
Endometrial cancers are the most common gynecologic malignancies of the female urogenital tract and the third most common cause of gynecologic cancer death. The incidence and mortality rates of endometrial carcinomas have increased in recent years with ~40,100 new cases and 7,470 deaths reported in 2008. This emphasizes the need for a detailed understanding of the molecular basis of endometrial carcinogenesis, which may lead to better diagnostic and therapeutic interventions for the disease.

The cytochrome P450 enzymes are a multigene family of constitutively expressed and inducible hemoproteins with a central role in the oxidative metabolism of a wide range of endogenous and exogenous compounds including many carcinogens (1, 2). CYP450 1B1 (CYP1B1) is a member of the CYP1 gene family and one of the major enzymes involved in the hydroxylation of estrogens and activation of potential carcinogens. CYP1B1 is constitutively expressed mainly in extrahepatic tissues including endometrium (3, 4). CYP1B1 gene is inducible by polycyclic aromatic hydrocarbons such as 2,3,7,8-tetrachlordibenzo-p-dioxin (5, 6) and is regulated by several key transcriptional factors, including aryl hydrocarbon receptor and estrogen receptor (7). CYP1B1 is involved in catalytic hydroxylation of estrogens to 4-hydroxy estrogens. The metabolic conversion of estrogens to 4-hydroxy estrogens induces DNA damage and has been postulated to play a major role in carcinogenesis (8–11). Although CYP1B1 is expressed in normal tissues, it is transcriptionally activated in several malignancies and is expressed at much higher levels in many tumors including colon, lung, kidney, and bladder (12). It shows particularly high expression in hormone-mediated cancers such as prostate, breast, and ovarian (8, 12–15). Additionally, due to differential CYP1B1 expression in the tumor microenvironment, CYP1B1 is an important tumor biomarker and a potential target for anticancer therapy (16–18).

Dysregulation of balance between cellular proliferation and apoptotic cell death leads to tumorigenesis. Progression of cells through the cell cycle is tightly regulated by interactions between cyclins, cyclin-dependent kinases (CDK; refs. 19, 20), and CDK inhibitors (21). The G1-S-phase transition is controlled by cyclin D1/Cdk4 and cyclin E/Cdk2. Cyclin E is essential for the control of the cell cycle at late G1 and early S phase (22). The cyclin E/Cdk2 complex is regulated by several CDK inhibitors including p27kip1 (21). p27kip1 levels are controlled by various mechanisms including phosphorylation-dependent ubiquitination and subsequent proteasomal degradation by S-phase kinase-associated protein 2 (Skp2; ref. 23). Skp2 is a ubiquitin protein ligase, which functions in proteasomal degradation of several other cell cycle regulatory proteins that contribute to cancer progression, including cyclin E, p53kip2, p21WAF1, and E2F1 (23), apart from proteins involved in signal transduction and transcription (24, 25). Overexpression of cyclin E and Skp2 contributes to the deregulated proliferation and genetic instabilities typical of cancer cells and is associated with various malignancies including endometrial cancers (26–29).

Apoptosis is a genetically controlled mechanism of cell death that plays a major role in tissue homeostasis and in many diseases.
Apoptosis can be induced through two signaling pathways: the intrinsic pathway or the extrinsic pathway. The intrinsic pathway is activated by intracellular signals from the mitochondria, which causes the release of cytochrome c that further leads to activation of initiator and effector caspases. The extrinsic pathway is initiated through the engagement of cell surface death receptors that belong to the tumor necrosis factor (TNF) superfamily (30, 31). TNF-related apoptosis-inducing ligand (TRAIL) is a member of the TNF superfamily that can initiate apoptosis by binding to the death receptors TRAIL-R1 (also called DR4) and TRAIL-R2 (also called DR5), thereby inducing caspase-mediated apoptosis. TRAIL protein preferentially induces apoptosis in transformed and tumor cells, but not normal cells, making it an attractive anticancer agent in various cancers (31, 32).

In the present study, we explored the role of CYP1B1 in the pathogenesis of endometrial carcinomas. In human endometrial cancers, it has been reported that 4-hydroxyestradiol levels are elevated in comparison with normal tissues and administration of this metabolite induces endometrial adenocarcinoma in mice (11). This supports the idea that CYP1B1 may play a key role in endometrial carcinomas. Despite the recognition of this enzyme as a key player in carcinogenesis, the detailed molecular mechanisms underlying CYP1B1-mediated endometrial carcinogenesis have never been investigated. In the present study, immunohistochemical staining showed that CYP1B1 expression is up-regulated in endometrial cancers. To understand the functional significance of CYP1B1 overexpression with regard to tumorigenesis, we depleted the gene in endometrial cancer cell line by RNA interference and monitored cellular proliferation, apoptosis, and invasion. To understand the molecular basis of the role of CYP1B1 in endometrial carcinomas, we profiled the expression of key genes of the biological pathways involved in transformation and tumorigenesis and identified several novel targets of CYP1B1. We found several cell cycle, apoptotic, and cell adhesion components that are potentially regulated by this hemoprotein.

### Materials and Methods

**Cell lines and cell culture.** The human endometrial cancer cell lines KLE, Ishikawa, HEC-1-B, and RL95-2 were obtained from the American Type Culture Collection. The KLE, Ishikawa, and RL95-2 cell lines were maintained in modified DMEM/F-12 (American Type Culture Collection) supplemented with 10% fetal bovine serum. The HEC-1-B cell line was cultured in Eagle's MEM supplemented with 10% fetal bovine serum. All cell lines were cultured in a humidified incubator (5% CO₂) at 37°C.

**Reverse transcription and real-time PCR.** Total RNA was isolated from cultured cells using the RNeasy mini kit (Qiagen) according to the manufacturer’s directions. cDNAs were synthesized with oligo(dT) primers by use of a SuperScript first-strand cDNA synthesis kit (Invitrogen) according to the manufacturer’s protocol. Gene expression was assessed by real-time quantitative PCR (RT-QPCR) using an Applied Biosystems 7500 Fast Sequence Detection System and gene-specific TaqMan assay kits (Applied Biosystems). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene was used as endogenous control to normalize expression data. Thermal cycling conditions included 95°C for 20 s, 40 cycles of 95°C for 3 s, and 60°C for 30 s according to the TaqMan Fast Universal PCR protocol. Each sample was analyzed in quadruplicate. The comparative Ct (threshold cycle) method was used to calculate the relative changes in gene expression in the 7500 Fast Real-time PCR System. The results are representative of three independent experiments. Data are shown as mean ± SE.

For real-time PCR-based expression profiling of a set of genes of a particular pathway, pathway-specific PCR arrays (cell cycle, apoptosis, and cancer pathwayfinder; SABiosciences) were used as per manufacturer’s instructions. Briefly, 1 μg of each RNA was reverse transcribed using the Reaction Ready First-Strand cDNA synthesis kit (SABiosciences) followed by PCR amplification using pre dispensed gene-specific primers and quantitative PCR master mix (SABiosciences) containing SYBR Green and reference dyes. Thermal cycling was done on an ABI Prism 7500 Fast thermal cycler using the following conditions: 95°C for 10 min, 40 cycles of 95°C for 15 s, 60°C for 1 min.

**Figure 1.** CYP1B1 is up-regulated in endometrial cancer. Immunohistochemical staining of CYP1B1. Endometrial tissue microarray was immunostained with CYP1B1 antibody to examine CYP1B1 expression in endometrial cancer tissues and normal adjacent tissues from the same patients. The slides were counterstained with hematoyxin. A and B, representative examples of CYP1B1 immunostaining. Magnification, ×200. Right, CYP1B1 staining in tumor tissues; left, corresponding normal adjacent tissues.
Abcam; Skp2 (4358), TRAIL (3219), and p27Kip1 (3686) were from Cell antibodies for Western blot analysis were purchased from carried out using standard protocols. The CYP1B1 (ab32649) and cyclin E kit (Pierce) according to the manufacturer’s instructions. Total protein cocktail (Roche). Protein estimations were done using a BCA Protein assay done using the ImmunoCruz Staining System (Santa Cruz Biotechnology) as incubated overnight with anti-CYP1B1 antibody (Abcam). The staining was by microwaving the slides in 10 mmol/L sodium citrate buffer. Slides were cancer. The slides were deparaffinized and antigen retrieval was carried out paraffin-embedded endometrial cancer tissue arrays (EMC241 and EMC962; U.S. Biomax). These included 12 cases of endometrial carcinoma with transfected using Oligofectamine (Invitrogen) with small interfering RNA transfected 24 h before transfection. At 30% to 50% confluence, cells were silencing (siRNA) duplexes specific for human CYP1B1 (Qiagen) or control non-silencing (NS) siRNA. Initially, four different sets of siRNA duplexes at different concentrations were tested to evaluate the target specificity and knockdown efficiency. The siRNA duplex showing the most efficient CYP1B1 knockdown was used for further experiments at 50 nmol/L concentration. The following are the sequences of CYP1B1 siRNA sense-r(GCAUGAUGCGCAACUUCUU)dTdT and antisense-r(AAGAAGUUGCCAUCAUGC)TdT4G. Also, mock control was included where cells were treated with Oligofectamine alone. The siRNA experiment was carried out for 72 h. Total RNA and proteins were analyzed by RT-QPCR and Western blotting.

**Cell proliferation assay.** For cell proliferation assay, KLE cells were seeded in 96-well microplates at a density of 5 × 10^3 per well 24 h before transfection. After transfection, cell viability was determined at 24, 48, 72, and 120 h by using the CellTiter 96 AQueous One Solution Cell Proliferation Assay kit (Promega) according to the manufacturer’s protocol. Absorbance at 490 nm was measured with a kinetic microplate reader (Spectra MAX 190; Molecular Devices) and was used as a measure of cell number. Experiments were done in quadruplicate and repeated three times.

**Cell cycle analysis.** Cell cycle analysis was done 72 h post-transfection. The cells were harvested, washed with cold PBS, and resuspended in the nuclear stain 4,6-diamidino-2-phenylindole (Beckman Coulter). Stained cells were immediately analyzed with a flow cytometer (Cell Lab Quanta SC; Beckman Coulter).

**Apoptosis assay.** For measuring apoptosis, transfected cells were dual stained with the viability dye 7-amino-actinomycin D and Annexin V-FITC using Annexin V-FITC/7-amino-actinomycin D kit (Beckman Coulter) according to the manufacturer’s protocol. Stained cells were immediately analyzed by flow cytometry (Cell Lab Quanta SC; Beckman Coulter).

** Invasion assay.** Control cells (mock or NS siRNA-treated) or CYP1B1 siRNA-treated cells were analyzed for invasion/migration through Matrigel (BD Biosciences) according to the manufacturer’s protocol. Briefly, 48 h post-transfection, cells were placed in Matrigel inserts or control inserts at 1 × 10^5/mL in serum-free medium and were allowed to migrate for 20 h at 37°C. Nonmigrating cells were removed from the top of the filter by scrubbing with a cotton swab. Cells that migrated were fixed and stained with a Hema 3 kit (Fisher Chemicals). The number of cells that migrated to the bottom side of the insert were counted manually and are presented as percentages of invasion.

**Statistical analysis.** Statistical analysis was done using StatView version 5.0 for Windows as needed. Student’s t test was used to compare the different groups. P values < 0.05 were regarded as statistically significant.
Results

**CYP1B1 is up-regulated in endometrial cancer.** To examine CYP1B1 expression in human endometrial cancers, endometrial cancer tissue array consisting of 48 cases of endometrial cancers and noncancerous cases was immunostained with CYP1B1 antibody. This included 12 cases of endometrial carcinoma with corresponding normal adjacent tissues and 36 other cases of endometrial cancer with 12 normal endometrial tissues. Compared with normal adjacent control tissues, CYP1B1 expression was found to be up-regulated in ~50% of endometrial adenocarcinomas. Representative examples of CYP1B1 immunohistochemistry are shown in Fig. 1A and B. Also, 14 of 36 cases showed strong CYP1B1 positivity compared with weak or moderate staining observed in normal endometrial tissues, suggesting an up-regulation of this monoxygenase in endometrial cancers.

**CYP1B1 affects cellular proliferation of endometrial cancer cells.** To explore the functional role of CYP1B1 in endometrial carcinogenesis, endometrial cancer cell lines were used. The relative expression of CYP1B1 was checked in human endometrial cancer cell lines KLE, Ishikawa, HEC-1-B, and RL95-2 using RT-QPCR (Fig. 2A). Ishikawa cells are well-differentiated and lack tumorigenic activity in nude mice (33), whereas RL-95 (34) and HEC-1-B (35) are moderately differentiated endometrial carcinoma cell lines. The KLE cell line is derived from undifferentiated endometrial cancer with defective estrogen receptor and possess tumorigenic activity in nude mice (36). It was observed that all the endometrial cancer cell lines show a high expression of CYP1B1 (Fig. 2A). However, the KLE cell line exhibits the highest expression level of this CYP isoform and was used for further functional studies.

To assess the role of CYP1B1 in endometrial cancers, we used a siRNA-mediated approach to knockdown endogenous CYP1B1 in KLE cells followed by functional assays. CYP1B1-specific siRNA resulted in ~80% depletion of endogenous CYP1B1 (Fig. 2B and C). We monitored cellular proliferation after CYP1B1 knockdown in KLE cells and found that cell viability significantly decreased in a time-dependent manner in CYP1B1 siRNA-treated cells compared with the control cells (Fig. 2D), suggesting that the attenuation of CYP1B1 expression has an antiproliferative effect in endometrial carcinomas.

![Figure 3: CYP1B1 affects cell cycle progression of endometrial cancer cells and regulates the expression of several cell cycle–related genes. Cell cycle distribution of endometrial cancer cells on CYP1B1 knockdown. Cell cycle progression was analyzed in KLE cells treated with either NS siRNA (A) or CYP1B1 siRNA (B) by flow cytometry of 4',6-diamidino-2-phenylindole–stained cells. Representative result. C, cell cycle genes affected by depletion of CYP1B1. CYP1B1-regulated genes were identified by cDNA expression profiling of cell cycle–related genes. The relative expression of the indicated genes was assessed in control and CYP1B1 siRNA-treated or NS siRNA-treated KLE cells by RT-QPCR analysis using TaqMan gene expression assays. Data were normalized to GAPDH. Mean ± SE. Relative expression of HERC5 is plotted separately (right). D, Western blot analysis of cell cycle components altered by CYP1B1 knockdown. Protein levels of cyclin E, Skp2, and p27 were assessed in NS siRNA-transfected or CYP1B1 siRNA-transfected KLE cells by immunoblotting with the respective antibodies. GAPDH was used as an internal loading control.]
CYP1B1 affects cell cycle progression of endometrial cancer cells and regulates the expression of several cell cycle–related genes. Following transfections with CYP1B1 siRNA in KLE cells, the effect of the knockdown on the cell cycle distribution was evaluated by flow cytometry. Representative cell cycle profiles in NS siRNA-treated or CYP1B1 siRNA-treated cells are shown in Fig. 3A and B, respectively. It was observed that the CYP1B1 depletion leads to a significant ~8% to 10% increase in the proportion of cells in the G0-G1 phase of the cell cycle, whereas the S-phase population decreases from 12% to 6%. This cell cycle distribution suggests that CYP1B1 knockdown leads to G0-G1 arrest in endometrial cancer cells. Also, cell cycle analysis showed an increase in the subdiploid (sub-G0-G1) population from 3% to 6%, suggestive of an increase in apoptosis after the knockdown.

To understand the mechanistic basis of cell cycle alterations induced by CYP1B1 knockdown in KLE cells, we profiled the expression of key cell cycle–related genes in CYP1B1 siRNA-treated and NS siRNA-treated cells and mock control and identified several cell cycle genes that are potentially regulated by CYP1B1 (Fig. 3C). Cyclin E1 (CCNE1) and SKP2 were down-regulated ~2- to 3-fold on treatment of endometrial cancer cells with CYP1B1 siRNA (Fig. 3C).

CYP1B1 influences apoptosis in endometrial carcinomas and regulates the expression of several apoptotic components. To further understand the role of CYP1B1 up-regulation in endometrial carcinomas, we also evaluated the effect of CYP1B1 knockdown on apoptosis in KLE cells. Apoptosis was examined in control NS siRNA-treated cells (Fig. 4A) or CYP1B1 siRNA-treated cells (Fig. 4B) by flow cytometric analysis of Annexin V-FITC/7-amino-actinomycin D–stained cells. The apoptotic cell fractions...
(early apoptotic + apoptotic) were significantly increased in CYP1B1-depleted cells (7% + 12%) compared with control cells (3% + 5%) with a concomitant decrease in the viable cell population (Fig. 4A and B).

To gain insight into the molecular mechanisms of the role of CYP1B1 in endometrial carcinomas, we initially probed the expression of key pathways involved in transformation and tumorigenesis using human cancer pathwayfinder PCR array (SABiosciences) on CYP1B1 knockdown and identified several apoptosis-related genes as potential CYP1B1 targets in addition to cell cycle and adhesion genes (data not shown). Apoptotic pathway-focused gene expression profiling confirmed IFN-β (IFNβ), granzyme A (GRZA), and (TRAIL)/TNF (ligand) superfamily, member 10 (TNFSF10) as novel CYP1B1-regulated genes (Fig. 4C). siRNA-mediated knockdown of CYP1B1 leads to ~13- and ~17-fold increase in expression of IFNβ and GRZA, respectively. Interestingly, CYP1B1 depletion leads to a large increase in expression of TRAIL at both mRNA and protein levels (Fig. 4C and D).

CYP1B1 influences the invasive properties of endometrial carcinomas and regulates the expression of melanoma cell adhesion molecule (MCAM). To examine the role of CYP1B1 in invasion of endometrial carcinomas, we evaluated the effects of CYP1B1 knockdown on the invasive properties of KLE cells in an in vitro Matrigel invasion assay (Fig. 5A). Cells treated with CYP1B1 siRNA showed significantly less (9%) invasion than NS control oligonucleotide (26%)–treated cells or mock control (23%; Fig. 5A). This indicates that CYP1B1 can modulate the invasive properties of endometrial carcinomas and its up-regulation may be linked to invasion and metastatic behavior in endometrial cancers. Further, we identified melanoma cell adhesion molecule (MCAM) as a novel target of CYP1B1 as MCAM expression decreased consistently (~3-fold) on CYP1B1 knockdown (Fig. 5B).

Discussion

It is recognized that many cytochrome P450 enzymes are differentially expressed in the tumor microenvironment and are therefore being considered as potential targets for the development of cancer therapeutics. Here we investigated the functional role of CYP1B1 in endometrial carcinogenesis by gene depletion followed by functional assays and limited gene expression profiling.

Immunohistochemical staining of endometrial carcinomas showed that CYP1B1 is up-regulated in a significant proportion of endometrial cancers. Also, moderate to weak CYP1B1 staining was observed in some of the normal tissues as CYP1B1 is known to be expressed in normal endometrium (3, 4). CYP1B1 catalyzes the hydroxylation of estrogens to 4-hydroxy estrogens that induces cellular damage (8–11). This estrogen metabolite has been shown to induce endometrial adenocarcinoma in mice (11), and in endometrial cancers, 4-hydroxyestradiol levels have been reported to be elevated in comparison with normal tissues.

Disregulated cellular proliferation is one of the hallmarks of cancer. Aberrations in the normal cycling of cells underlie uncontrolled cellular proliferation. Here we observed that CYP1B1 depletion in endometrial carcinoma cells leads to decreased cellular proliferation and induces G0-G1 cell cycle arrest concomitant with a decrease in S phase. Significantly in our present study, we found that CYP1B1 knockdown in endometrial cancer cell line leads to altered expression of cell cycle regulatory components that have a role in G1-S-phase transition of the cell cycle. This included down-regulation of expression of cyclin E1 and Skp2 and up-regulation of the CDK inhibitor p27kip1.

Cyclin E1 plays an essential role in the G1-S-phase transition of the cell cycle as well as the initiation of DNA replication (22). Cyclin E overexpression has been observed previously in endometrial carcinomas (27, 37, 38) and high levels correlate with increased tumor aggression and poor prognosis (39). Skp2 is involved in proteasomal degradation of phosphorylated CDKN1B/p27kip1 and also regulates cyclin E, p57kip2, p21WAP1, and E2F1, thereby controlling G1-S-phase transition of the cell cycle (23). It has been reported previously that a progressive derailment of cell cycle regulators including cyclin E, cdk2, p21, and p27 occur in endometrial carcinogenesis (27). Endometrial carcinomas are also associated with increased Skp2/decreased p27 levels (28, 29). Our present results indicate that CYP1B1 regulates cyclin E1, Skp2, and p27 and thereby affects G1-S-phase transition of the cell cycle. We observed G0-G1 arrest concomitant with increased p27 levels that is known to be up-regulated in quiescent cells.

Expression profiling of cell cycle–related genes also identified MCM4 as a CYP1B1–regulated gene. The protein encoded by MCM4...


17. McFadyen MC, McLeod HL, Jackson FC, Melvin WT, Cancer Research.
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