Endometrial cancer (EC) is a steroid hormone-dependent cancer. Uridine 5′-diphospho-glucuronosyltransferase enzymes conjugate and detoxify endogenous and exogenous steroid hormones and environmental carcinogens. Among these enzymes, the function of UGT2B17 is unknown except for glucuronidation. The messenger RNA expression of UGT2B17 and myeloid cell leukemia-1 (Mcl-1) was significantly increased in EC tissues compared with matched normal endometrial tissues. Therefore, we focused on the function of UGT2B17 in EC. A total of nine patients with confirmed EC were enrolled in this study to investigate the expression of UGT2B17 and target genes. EC cell lines were used for functional tests including cell growth, invasion, apoptosis and cell cycle analyses. To find the target genes of UGT2B17, we performed microarray analysis to see which genes were upregulated or downregulated by UGT2B17-transfected cells. Functional analysis showed decreased numbers of viable cells and increased numbers of apoptotic cells in si-UGT2B17-transfected Ishikawa cells. Among microarray target genes, Mcl-1 was significantly downregulated in si-UGT2B17-transfected cells. We also found upregulation of Puma protein, a target of Mcl-1, in si-UGT2B17-transfected cells. This is the first report to show that UGT2B17 and Mcl-1 expression are upregulated in EC tissues and that UGT2B17 depletion induces inhibition of cell growth and apoptosis in EC cells through Mcl-1 downregulation.

Introduction

Endometrial cancer (EC) is a common gynecological cancer in the USA (1). The metabolism of estrogens has been thought to play an important role in the development and pathogenesis of EC (2). Estrogens are activated by biosynthetic enzymes (phase I) and detoxified by conjugation enzymes (phase II) (2). The phase II conjugation reactions include glucuronidation, sulfation and glutathione and amino acid conjugation (3). Glucuronidation is catalyzed by uridine 5′-diphospho-glucuronosyltransferase enzymes (4). After conjugation by glucuronidation, the derivatives are excreted through the bile or urine. UGT proteins generally have been categorized into four subfamilies (UGT1A, UGT2A, UGT2B and UGT3) based on homology (4,5). The UGT1A gene complex is located on chromosome 2q37 and encodes nine members of the UGT1A family (6). The UGT2A and UGT2B genes are located on chromosome 4q13–4q21.1 (5,6). The UGT2A subfamily includes three members and UGT2B subfamily includes 12 members (6). The UGT3 family was recently identified and members of the UGT3 family are ~30% homologous to those of other UGT families (6). With regards to UGT proteins and EC, some investigators have performed polymorphism studies and found that some UGT polymorphisms were related to EC risk (7,8). It has been well understood that UGT proteins play an important role in glucuronidation of endogenous and exogenous compounds including steroid hormones. Therefore, the function of UGT proteins has focused on pharmacogenetics in breast cancer treatment with tamoxifen (selective estrogen-receptor modulator) (9,10). Conversely, the function and expression of UGT protein are not well understood in EC. Recently Nakamura et al. (11) investigated the expression level of UGT1A and UGT2B messenger RNA (mRNA) in human normal tissues and various cancer cells. In their study, UGT1A5, UGT2B4, UGT2B7, UGT2B11, UGT2B15 and UGT2B17 mRNA was expressed in normal uterus (11), but only UGT2B17 mRNA expression was expressed in EC Ishikawa cells (11). Several UGT enzymes are regulated by estrogen level but the expression of UGT2B17 mRNA was not stimulated after estradiol (E2) treatment in MCF-7 cells (12). Recently, our laboratory did a functional analysis of CYP1B1, which is also an estrogen metabolic enzyme, in EC and found that CYP1B1 upregulation plays a crucial role in endometrial carcinogenesis by targeting multiple pathways, suggesting that CYP1B1 inhibition in EC could be a useful therapeutic approach (13). This evidence suggested to us that UGT2B17 function may be independent from estrogen regulation and may play a role in endometrial oncogenesis. To test this hypothesis, we first used real-time reverse transcription–polymerase chain reaction (RT–PCR) to confirm the expression of UGT2B17 in EC and adjacent endometrial normal tissues using paired samples. It was found that the mRNA expression of UGT2B17 was significantly increased in EC tissues compared with matched adjacent normal tissues (8/9 = 89%). We then examined the expression of UGT2B17 in several EC cell lines (KLE, HEC-1-B and Ishikawa cells) in order to choose one best suited for functional analysis. Since the expression of UGT2B17 was observed only in Ishikawa cells, we used Ishikawa cells for further experiments (MTS, apoptosis, cell cycle and invasion assays) to identify the function of UGT2B17. In order to examine the function of UGT2B17 in EC cells, we knocked down its expression using a siRNA technique and compared the effects between si-‘negative control’ and si-UGT2B17-transfected Ishikawa cells. We also performed microarray analysis to identify genes related to UGT2B17 expression.

Materials and methods

Clinical samples

A total of nine patients with pathologically confirmed EC were enrolled in this study (University of California, San Francisco). The pathology of all the patients was endometrioid adenocarcinoma. Patient information is as follows: stage IA (5), stage IB (2) and stage IIA (2) and grade I (7) and grade II (2). Stage information was according to International Federation of Gynecology and Obstetrics (FIGO) stage. Samples were obtained from the patients after written informed consent was obtained at the University of California, San Francisco.

Cell culture

EC cells (KLE, HEC-1-B and Ishikawa cells) were maintained in D-MEM/F-12 media (4.5 g/l glucose) supplemented with 10% fetal bovine serum, 1× penicillin/streptomycin and incubated at 37°C in a humidified incubator with 5% CO2.

RNA and protein extraction

RNA was extracted from cell lines using a QiAamp RNA kit (Qiagen, Valencia, CA). Tissue total RNA was extracted using a combination of TRIzol reagent (Invitrogen, Carlsbad, CA) and RNeasy columns (Qiagen). Fresh EC and adjacent normal endometrial tissues were homogenized in 1 ml TRIzol

Abbreviations: EC, endometrial cancer; mRNA, messenger RNA; Mcl-1, myeloid cell leukemia-1; PARP, poly ADP-ribose polymerase; RT–PCR, reverse transcription–polymerase chain reaction; si-NC, si-negative control; TRAIL, tumor necrosis factor-related apoptosis-inducing ligand.
To digest DNA, the Qiagen RNase-Free DNase kit was used. RNA quality was assessed by the NanoDrop ND-1000 (NanoDrop Technologies, Wilmington, DE) spectrophotometer. Extracted RNA was stored at –80°C until used. Cells were lysed with RIPA buffer (Pierce, Brefiennes, France) containing protease inhibitors (Sigma, St Louis, MO). Protein quantification was done using a BCA protein assay kit (Pierce).

Knockdown of UGT2B17 mRNA in Ishikawa cells

Ishikawa cells express estrogen receptors and progesterone receptors. However, according to previous reports, proliferation of Ishikawa cells was not promoted after estrogen treatment (14). Therefore, we did not use estrogen for culture of Ishikawa cells. Ishikawa cells were transfected with UGT2B17 siRNA (si-UGT2B17, Invitrogen) or negative control siRNA [si-negative control (si-NC, Invitrogen) according to the manufacturer’s instructions. Briefly, cells were grown in six-well plates and transfected individually with three siRNAs [HSS144421 (no. 1), HSS144422 (no. 2) and HSS144423 (no. 3)] at a concentration of 200 pmol/well. The siRNAs sequences are as follows: siRNA-no. 1, forward: 5′-AACCGCAUGCAGGACGCAUC-3′/reverse: 5′-UGAUUGCUUCUCUGCGACAGGUUGCUU-3′; siRNA-no. 2, forward: 5′-CUUCACAGACGCUUUAUUAUGC-3′/reverse: 5′-GACUUAUAUAUAACGCUGUGAGAG and siRNA-no. 3, forward: 5′-AAACAGAGAAGGCAGAGCUCA-3′/reverse: 5′-UCGUUAAGUCUCGGCCUCUCUCGUGU-3′. Transfection was performed with X-tremeGene siRNA Transfection Reagent (Roche Diagnostics, Basel, Switzerland).

Cell viability assay

Cell viability was measured with MTS (CellTiter 96 Aqueous One Solution Cell Proliferation Assay; Promega). Data are the mean ± SD of six independent experiments.

Apoptosis and cell cycle analysis

Cells (si-NC and si-UGT2B17) were trypsinized and washed once in complete medium centrifuged at 2000 r.p.m. for 5 min at 4°C and resuspended in ice-cold 1× binding buffer. Annexin V-FITC solution (10 l) and 7-aminoactinomycin D viability dye (20 l) were added to 100 l of the cell suspensions. After incubation for 15 min in the dark, 400 l of ice-cold 1× binding buffer was added. The apoptotic distribution of the cells in each sample was then determined using a fluorescence-activated cell sorting (Cell Lab QUANTA SC; Beckman Coulter, Fullerton, CA). The various phases of cells were determined using a fluorescence-activated cell sorting (Cell Lab QUANTA SC; Beckman Coulter, Fullerton, CA) according to the manufacturer’s instructions. Bivariate analyses were performed with a scatter plot with the x-axis representing the binding of the Annexin V-FITC Alexa Fluor 488, the y-axis representing the binding of 7-aminoactinomycin D, and a third axis representing the forward light scatter (FSC) and the side scatter (SSC).

Cell invasion assay

Cell invasion assay was performed with six-well BD Biocoat Matrigel invasion chambers as described previously (BD Biosciences, Bedford, MA). The cells (si-NC and si-UGT2B17) were resuspended to the upper chamber in triplicate. Cells migrating through the membrane were stained with HEMA3 (Fisher Scientific Company, Kalamaizo, MI) and counted with a microscope. Five random fields were chosen for each membrane, and the results were expressed as migrated cells per field.

Complementary DNA microarray and quantitative real-time RT–PCR in Ishikawa cells

Apoptosis-related genes were profiled with the Human RT² Profiler™ PCR Array (http://www.superarray.com/microarrays.php). The RT² first strand kit, which includes a proprietary buffer to eliminate any residual genomic DNA contamination in cell line RNA samples, was used to convert mRNA into complementary DNA. The manufacturer’s instructions were strictly followed. To analyze the PCR Array data, we used an MS-excel sheet from the manufacturer’s website. The PCR Array Data Analysis Web Portal was used to perform several calculations as follows: scatter plots, three-dimensional profiles and a volcano plot. Based on the scatter plot data (relative expression comparison between si-negative control-transfected and si-UGT2B17-transfected cells), we selected candidate genes whose expression levels were increased or decreased in si-UGT2B17-transfected cells compared with si-negative control cells (fold cutoff was 3.5). Data was normalized based on correcting all Ct (threshold cycle) values for the average Ct values of several constantly expressed housekeeping genes present on the array. Quantitative real-time RT–PCR was performed in triplicate to confirm the results of microarray analysis with an Applied Biosystems Prism 7500 Fast Sequence Detection System using TaqMan universal PCR master mix according to the manufacturer’s protocol (Applied Biosystems, Foster City, CA). The TaqMan probes and primers were purchased from Applied Biosystems. Human glyceraldehyde 3-phosphate dehydrogenase was used as an endogenous control. Levels of mRNA expression were determined using the 7500 Fast System SDS software version 1.3.1 (Applied Biosystems).

Western blotting

Total protein (20 l) was used for western blotting. Samples were resolved in 4–20% Precise Protein Gels (Pierce) and transferred to polyvinylidene difluoride membranes (Amersham Biosciences, Fairfield, CT). The membranes were immersed in 0.3% skim milk in Tris-buffered saline containing 0.1% Tween 20 for 1 h and probed with primary polyclonal and monoclonal antibody against poly ADP-ribose polymerase (PARP) (#9532, Cell Signaling Technology, Denver, MA, USA), cleaved PARP (#9546, Cell Signaling Technology), Bax (#2727, Cell Signaling Technology), Bcl-2 (#851097; BD Biosciences), Puma (#4976; Cell Signaling Technology), myeloid cell leukemia-1 (Mcl-1) (#4572; Cell Signaling Technology), Akt (#4691; Cell Signaling Technology), phosphorylating Akt (#4060; Cell Signaling Technology) and beta-actin (#49678; Santa Cruz) (14). Membranes were washed in Tris-buffered saline containing 0.1% Tween 20 and labeled with horseradish peroxidase-conjugated secondary anti-mouse or anti-rabbit antibody (Cell Signaling Technology). Proteins were enhanced by chemiluminescence (GE Healthcare) for visualization. The protein expression levels are expressed relative to beta-actin levels.

Statistical analysis

All statistical analyses were performed using StatView (version 5; SAS Institute, Cary, NC). A P-value of <0.05 was regarded as statistically significant.

Results

UGT2B17 and Mcl-1 expression in EC and adjacent normal endometrial tissues

UGT2B17 expression was significantly higher in EC tissues compared with adjacent normal endometrial tissues in paired tissue samples (8/9 = 89%) (Figure 1). Mcl-1 expression was increased in four of nine tissue samples (Figure 2).

UGT2B17 expression in EC cell lines and normal endometrial tissues

We analyzed UGT2B17 mRNA expression in three EC cell lines (KLE, HEC-1-B and Ishikawa cells). We did not detect UGT2B17 mRNA expression in KLE and HEC-1-B cells. Only Ishikawa cells exhibited UGT2B17 mRNA expression (Figure 3A).

UGT2B17 expression in Ishikawa cells (relative expression: 28) was high compared with endometrial normal tissues (relative expression: 1.0, 1.5) (Figure 3A).

siRNA transfection, MTS, invasion and fluorescence-activated cell sorting in Ishikawa cells

As UGT2B17 mRNA expression was found only in Ishikawa cells, we knocked down the mRNA of UGT2B17 in Ishikawa cells using a siRNA technique. We used three different si-UGT2B17s (no. 1, no. 2 and no. 3), among which si-UGT2B17 RNA no. 3 (HSS144423) was most effective in decreasing the expression of UGT2B17 in Ishikawa cells (Figure 3B). The knockdown effect was also confirmed by western blot (Figure 3C). Therefore, we used si-UGT2B17 no. 3 (HSS144423) for further experiments with Ishikawa cells (MTS, invasion and fluorescence-activated cell sorting) (Figure 4).

We found inhibition of cell growth with si-UGT2B17 transfection by MTS assay (Figure 4A). However, there were no significant differences in invasion assays between si-NC- and si-UGT2B17-transfected cells (Figure 4B). We found a significantly higher number of apoptotic cells in si-UGT2B17-transfected cells compared with si-NC cells (P < 0.05) (Figure 4C and D). The mean cell number in the S and G2/M phases of the cell cycle was also significantly lower in si-UGT2B17-transfected cells, suggesting that si-UGT2B17 induced G1 arrest in Ishikawa cells (Figure 4E). Apoptosis and cell cycle assay was done at 48 h after 72 h of siRNA (si-NC or si-UGT2B17) transfection because the proliferation in si-UGT2B17-transfected Ishikawa cells was significantly inhibited at this point (MTS, 48 h).

Complementary DNA microarray, quantitative real-time RT–PCR and western blotting in si-UGT2B17-transfected cells

We focused our study on apoptosis genes because we found growth inhibition and increased apoptosis in si-UGT2B17-transfected cells. We initially profiled apoptosis-related genes with the Human RT² Profiler™ PCR Array.
Profiler™ PCR Array. A human apoptosis complementary DNA microarray, which included 87 genes, was used in this study. A cluster gram is shown in Figure 4. Genes found to be differentially expressed were clustered using hierarchical cluster analysis to visualize the pattern of gene expression for each group. The heat map indicates that red represents highly expressed genes, whereas green represents under expressed genes; the black color on the heat map represents equal expression relative to control. Based on these microarray data, seven genes were identified as being either upregulated (six genes) or downregulated (one gene) in si-UGT2B17-transfected cells compared with si-NC-transfected cells using this microarray (threshold, 2.5) (Figure 5A; supplementary Table S1 is available at Carcinogenesis Online). Among the identified candidate genes, Mcl-1 mRNA expression was confirmed by real-time RT–PCR. Namely, the expression of Mcl-1, an anti-apoptotic gene, was significantly downregulated by si-UGT2B17 transfection (Figure 5B). We also confirmed protein expression level of this gene and found it to be significantly decreased in UGT2B17 knockdown (Figure 6).

**Fig. 1.** Expression of UGT2B17 mRNA in EC and adjacent normal uterus tissues. Expression levels of UGT2B17 mRNA in EC tissues and adjacent normal endometrial tissues (nine paired samples).

**Fig. 2.** The mRNA expression level of Mcl-1 in EC and matched adjacent normal uterus tissues. Expression levels of Mcl-1 mRNA in EC tissues and adjacent normal endometrial tissues (nine paired samples).
**Fig. 3.** Expression of UGT2B17 mRNA in EC cells (A) and the effect of si-UGT2B17 on Ishikawa cells (B and C). (A) UGT2B17 mRNA expression in three EC cell lines. (B) Effect of si-UGT2B17 (no. 3) on the expression of UGT2B17 mRNA (B) and protein (C) in Ishikawa cells.

**Fig. 4.** Effect of si-UGT2B17 on Ishikawa cell growth (A), invasion (B), apoptosis (C and D) and cell cycle (E). (A) MTS assay, (B) invasion assay, (C and D) apoptosis assay, Annexin V-FITC and 7-aminoactinomycin D were measured by flow cytometry. Representative results are shown in Figure 3D. Data are the mean ± SD of four independent experiments. (E) Cell cycle analysis. Enumeration of the various phases of the cells was determined using a DNA stain such as 4',6-diamidino-2-phenylindole. G0/G1, S and G2/M populations were measured using fluorescence and contrasted against cell volume. Data are the mean ± SD of four independent experiments.
To confirm increased apoptosis, we performed western analysis of PARP and cleaved PARP. As shown in Figure 5, significantly increased expression of cleaved PARP was found in si-UGT2B17-transfected Ishikawa cells. The expression of Bax, Akt and phosphorylated Akt proteins were not changed, however, expression of Puma was increased in si-UGT2B17-transfected cells.

Discussion

The study of estrogen metabolic and conjugation enzymes have focused on the relationship between enzyme activity, polymorphism, cancer susceptibility and response to chemotherapy (8). Similar to other enzymes, there have been a number of polymorphism studies on UGT2B17 (15,16), and functional studies of UGT2B17 have been focused on the glucuronidation of endogenous and exogenous compounds including steroid hormones (17–19). UGT2B17 is expressed in the liver and various extrahepatic tissues including testis, uterus, prostate and lung (19). Recently Nakamura et al. (11) investigated the expression of the UGT2B family in various normal tissues and observed UGT2B17 mRNA expression in normal endometrial tissues. However, there have been no reports comparing the expression level of UGT2B17 in EC tissues and adjacent normal tissues. Therefore, we looked at UGT2B17 mRNA expression in EC Ishikawa cells as previously reported (11) and used these cells for functional analysis of UGT2B17.

The UGT family is involved in the glucuronidation of endogenous and exogenous steroid hormones and detoxification of environmental carcinogens, and hence, low UGT expression or activity may increase the risk of various cancers including EC (20). However, we observed that UGT2B17 mRNA expression was higher in EC tissues than in adjacent normal tissues in this study. Given that UGT2B17 mRNA was not regulated by estrogen level in breast cancer MCF-7 cells (12), we hypothesized that UGT2B17 might have other functions independent of estrogen, which promote EC. To test this hypothesis, we knocked down UGT2B17 mRNA in Ishikawa cells with an siRNA and observed its effects on cell growth, invasion, apoptosis and the cell cycle. In si-UGT2B17-transfected Ishikawa cells, the number of viable cells was significantly decreased compared with si-negative control-transfected cells. In addition, the number of cells in the apoptotic and G0/G1 phase was significantly increased after si-UGT2B17 transfection. The expression level of cleaved PARP was also significantly increased in si-UGT2B17-transfected Ishikawa cells compared with control cells. However, there was no significant change in the number of invasive cells after transfection of si-UGT2B17. Therefore, these data suggest that UGT2B17 may play a role in apoptosis and the cell cycle but not invasion ability.

In order to further investigate the molecular mechanisms of UGT2B17 function, we used microarrays to screen for apoptosis-related genes and found altered expression of several genes in
si-NC   si-UGT2B17

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<tr>
<th>Protein</th>
<th>si-NC</th>
<th>si-UGT2B17</th>
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<tr>
<td>Bax (20 kDa)</td>
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<td>PARP (116 kDa)</td>
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<td>Cleaved PARP (89 kDa)</td>
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<td>Mcl-1 (40 kDa)</td>
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<td>Puma (23 kDa)</td>
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<td>Bcl-2 (28 kDa)</td>
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<td>Akt (60 kDa)</td>
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<td>p-Akt (60 kDa)</td>
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<td>Beta-actin (45 kDa)</td>
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Fig. 6. Protein expression in si-NC-transfected or si-UGT2B17-transfected Ishikawa cells. The protein expression of Mcl-1 was significantly decreased in si-UGT2B17-transfected Ishikawa cells, whereas Puma protein expression was increased.

UGT2B17 and EC related kinase pathway is a target of sorafenib (mitogen activated protein kinase pathway). One of the mechanisms of apoptosis induction by sorafenib is Mcl-1 downregulation through reduced translation (21–25). Llobet et al. (34) found that EC cell death triggered by sorafenib was mediated by downregulation of Mcl-1. In this study, Mcl-1 expression was downregulated in UGT2B17-depleted cells. Therefore, UGT2B17-depleted cells are similar to those treated with sorafenib, with alterations of the target genes of Mcl-1. As expected, the expression of Puma was increased in UGT2B17-knockdown cells. As Akt and Mcl-1 have been shown to play an important role in TRAIL-induced apoptosis, we looked at Akt, phosphorylated-Akt and Mcl-1 expression (35). However, no relationship was found in western blot results suggesting that UGT2B17 itself may not affect the Mcl-1–Akt pathway.

We also examined Mcl-1 mRNA expression levels in EC tissues and matched adjacent normal endometrial tissues to see the relationship between UGT2B17 mRNA and Mcl-1 mRNA expression. Mcl-1 expression was increased in four of nine EC tissue samples. Among cancer tissue samples (n = 8) with increased UGT2B17 mRNA expression, Mcl-1 expression was also increased in four samples (50%) but not changed in two others. A tissue sample with decreased UGT2B17 expression in cancer similarly had decreased expression of Mcl-1. Thus, there may be a positive correlation between UGT2B17 and Mcl-1 mRNA expression. We also examined the relationship between UGT2B17 expression and clinical parameters such as stage and grade. However, we did not find a significant relationship. Since our sample number is relatively small, a larger study will be needed to look at the correlation between UGT2B17 expression and clinical parameters.

In conclusion, this is the first report to show that UGT2B17 and Mcl-1 expression is upregulated in EC tissues and that UGT2B17 knockdown inhibited cell growth and increased apoptosis in EC cells through Mcl-1 downregulation. However, additional studies will be required to reveal the exact molecular mechanisms involved in the regulation of Mcl-1 expression by UGT2B17. These findings also suggest that the expression level of UGT2B17 may be an endometrial tumor marker and silencing UGT2B17 may be beneficial as a therapeutic tool for EC. Since there are many UGT families, similar functional studies of these families have not been carried out. Therefore, the expression pattern of all UGT families in EC and adjacent normal tissues may be useful to predict them oncogenic or tumor suppressive effects.

Supplementary material

Supplementary Table S1 can be found at http://carcin.oxfordjournals.org/

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References


