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Catechol-*O*-methyltransferase-mediated metabolism of 4-hydroxyestradiol inhibits the growth of human renal cancer cells through the apoptotic pathway

Inik Chang, Jan Liu, Shahana Majid, Sharanjot Saini, Mohd S.Zaman, Soichiro Yamamura, Varahram Shahryari, Takeshi Chiyomaru, Guoren Deng, Rajvir Dahiya and Yuichiro Tanaka*

Department of Urology, San Francisco Veterans Affairs Medical Center and University of California at San Francisco, 4150 Clement Street, San Francisco, CA 94121, USA

*To whom correspondence should be addressed. Tel: +1 415 750 2031; Fax: +1 415 750 6639;
Email: yuichiro.tanaka@ucsf.edu

Long-term exposure to estrogen and its metabolites may play an important role in renal cell carcinogenesis. Catechol-*O*-methyltransferase (COMT) participates in the estrogen metabolism pathway by neutralizing toxic substances. Although reduced COMT activity has been suggested to be a risk factor for estrogen-associated cancers, no studies have investigated the biological significance of COMT in the pathogenesis of human renal cell cancers (RCCs). We initially found that COMT levels are significantly decreased in human RCC tissues and cells suggesting it plays a suppressive role in tumor development. However, transient overexpression of COMT has no functional effect on RCC cell lines. In contrast, when cells overexpressing COMT are treated with its substrate 4-hydroxyestradiol (4-OHE_2), growth is inhibited by apoptotic cell death. We also found that COMT overexpression combined with 4-OHE_2 induces upregulation of growth arrest- and DNA damage-inducible protein α (GADD45α). We further show that downregulation of GADD45α by a small interfering RNA-mediated approach inhibits cell death, indicating the essential role of GADD45α in the underlying mechanism of COMT action in response to 4-OHE_2. Finally, 4-methoxyestradiol fully reproduces the antiproliferative function of COMT with 4-OHE_2 by promoting GADD45α induction. Together, these findings show that COMT in the presence of 4-OHE_2 prevents RCC cell proliferation by enhancing apoptosis and that GADD45α plays a critical role in the COMT-mediated inhibition of RCC.

Introduction

In the USA, kidney cancer is among the 10 most common cancers in both men and women. It is estimated that 60 920 new cases and 13 120 deaths from the disease will occur in 2011 (1). Renal cell cancer (RCC) is the most frequent type of kidney cancer and ∼80% of RCC are clear cell adenocarcinomas, the remainder being papillary (∼15%), chromophobe (∼5%) and collecting duct carcinomas (<1%) (2). Genetic susceptibility, cigarette smoking, obesity, hypertension, chronic renal failure and occupational exposure to trichloroethylene have been considered as strong risk factors for RCC (1,2). There are almost twice as many RCC cases in men as in women, and men generally present with more severe malignancy, such as larger size, higher grade and higher stage of tumors than women. In addition, the mean age at diagnosis for men is slightly earlier and both overall and 5 years survival rate is also shorter than that of women (3,4). Although the reason for difference in rates of RCC between men and women remains unknown, there is substantial evidence suggesting that estrogen may be an additional etiological factor for RCC, especially in men. According to a report by Lipworth *et al.* (2), the average age at RCC diagnosis is in the early 60s and men at this age have much higher levels of estrogen compared with postmenopausal women of similar age (5,6). Renal carcinoma has been found in male patients who had received long-term estrogen therapy for treating prostatic carcinoma (7,8). However, no association of RCC risk with estrogen replacement therapy in women was observed (9). In the golden Syrian hamster, kidney tumors are normally produced only in males treated with synthetic estrogen and the induction of tumor can be blocked by the administration of estrogen antagonists (10,11).

In extrahepatic tissues including kidney, endogenous estrogens undergo oxidative metabolism catalyzed by cytochrome P450 1A1 and 1B1 to yield catechol estrogens, 2-hydroxyestradiol and 4-hydroxyestradiol (4-OHE_2), respectively. Of the two catechol estrogens, 4-OHE_2 is believed to be a critical intermediate in estrogen-induced cancers. It is further oxidized by metabolic redox cycling, and the resultant estradiol-3,4-quinones and reactive oxygen species have genotoxic and carcinogenic effects (12). 4-OHE_2 is also capable of inducing cytotoxicity in human mammary epithelial cells and activation of nuclear factor-kappaB and extracellular signal-regulated kinase/mitogen-activated protein kinase pathways (13).

Catechol-*O*-methyltransferase (COMT) is a phase II enzyme in the process of estrogen metabolism. It catalyzes *O*-methylation at either the 2-, 3-, 4-position of the catechol ring of a substrate and thus, methoxyestradiols (MEs) are produced from corresponding hydroxyestradiols (14). It has been suggested that COMT may prevent the development of estrogen-associated cancers by this metabolic *O*-methylation of endogenous catechol estrogens. It reduces the level of 4-OHE_2, thereby lowering the potential for genomic damage through DNA adduct formation or through oxygen radicals arising from quinone–semiquinone redox cycling of catechol estrogen (15,16). 2-ME, which is produced from 2-OHE_2 has antiproliferative, antiangiogenic and proapoptotic activity in multiple types of cancer (17). There is evidence for the protective role of COMT in estrogen-induced carcinogenesis. Administration of a COMT inhibitor, quercetin, increased the number of large renal tumors and the incidence of abdominal metastases in estradiol-treated hamsters (18,19). COMT also protects catechol estrogen-induced malignant transformation of human endometrial glandular cells by regulating oxidative stress (20). Salama *et al.* (21) showed that 2-ME produced by COMT overexpression ameliorates estradiol-induced proliferation of human uterine leiomyoma cells by disturbing microtubule dynamics, inhibiting steroid receptor signaling and reducing HIF-1α and CYP19 expression.

The association of COMT polymorphisms with the risk of various cancers of the breast, endometrium, prostate, bladder, liver, stomach and esophagus has been investigated. Recently, we found that a COMT polymorphic variant, which may alter messenger RNA (mRNA) stability and/or translation efficiency, is significantly associated with risk for RCC in men (22). Therefore in this study, we examined the expression level and functional significance of COMT in human RCC. Our results show that both the mRNA and protein levels of COMT are significantly decreased in RCC. Although overexpression alone has no obvious effect, COMT expression in the presence of 4-OHE_2 inhibits the proliferation of RCC cells, which results from increased apoptotic cell death. In addition, the critical role of 4-ME formation and subsequent growth arrest- and DNA damage-inducible protein α (GADD45α) induction as the underlying mechanism of COMT action were demonstrated.

Abbreviations: cDNA, complementary DNA; COMT, catechol-O-methyltransferase; FITC, fluorescein isothiocyanate; GADD45α, growth arrest- and DNA damage-inducible protein α; 4-OHE_2, 4-hydroxyestradiol; ME, methoxyestradiol; mRNA, messenger RNA; PCR, polymerase chain reaction; Q-PCR, quantitative PCR; RCC, renal cell cancer; siRNA, small interfering RNA.

Materials and methods

Cell lines and reagents

The immortalized human renal proximal tubule epithelial cell line, HK-2, renal cancer cell lines, Caki-1, ACHN and 769-P, and a prostate cancer cell line, LNCaP were obtained from the American Type Culture Collection (Manassas, VA). Keratinocyte serum-free medium, bovine pituitary extract and recombinant epidermal growth factor were purchased from Invitrogen (Carlsbad, CA). McCoy's 5A, Eagle's minimum essential medium with Eagle's balanced salts solution (EMEM), RPMI 1640, Opti-minimum essential medium and penicillin/streptomycin mixtures were obtained from the UCSF Cell Culture Facility. Phenol red-free McCoy's 5A and Eagle's minimum essential media were purchased from PromoCell (Heidelberg, Germany) and Invitrogen, respectively. Fetal bovine serum was a product of Atlanta Biologicals (Lawrenceville, GA). 4-OHE_2 and Ro 41-0960 were purchased from Sigma Chemical Co. (St Louis, MO). 4-ME was obtained from Steraloids (Newport, RI).

Cell culture

The HK-2 cell line was maintained in keratinocyte serum-free medium supplemented with 0.05 mg/ml bovine pituitary extract and 5 ng/ml recombinant epidermal growth factor. The Caki-1 and ACHN cell lines were grown in McCoy's 5A and Eagle's minimum essential medium, respectively. The 769-P and LNCaP cell lines were cultured in RPMI 1640 medium. All culture medium contained 10% fetal bovine serum and 100 μg/ml penicillin/streptomycin mixture. All cell lines were maintained at 37°C in a humidified atmosphere composed of 5% CO_2/95% air.

Establishment of RCC cell lines stably expressing COMT

Caki-1 and ACHN cells were transfected with pCMV6-ENTRY vector expressing the C-terminally Myc and Flag-tagged human membrane bound (MB)-COMT complementary DNA (cDNA) as well as empty pCMV6-ENTRY vector as a control (OriGene Techologies, Rockville, MD) using Fugene HD Transfection Reagent (Roche Diagnostics, Indianapolis, IN) according to the manufacturer's protocol. Stable clones were selected with 500 μg/ml of G418 (Invitrogen). Colonies resistant to G418 appeared within 2 weeks, and single colonies were picked and then expanded for another 3 weeks to make the original stock cells.

Quantitative reverse transcription–polymerase chain reaction

Total RNA from cultured cell lines was isolated using the RNeasy Mini Kit (Qiagen, Valencia, CA) and was converted into cDNA by using the iScript™ cDNA Synthesis Kit (Bio-Rad, Hercules, CA) according to the manufacturer's instruction. To assess gene expression, cDNAs were amplified with the TaqMan® Gene Expression Assays and TaqMan® Fast Universal polymerase chain reaction (PCR) Master Mix using the 7500 Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). The target genes and their Assay ID were as follows: COMT (Hs00241349_m1) and GADD45α (Hs00169255_m1). Beta-actin was utilized to normalize COMT expression in normal and RCC clear cell tissues and glyceraldehyde-3-phosphate dehydrogenase was used as an internal control unless stated otherwise. The relative change in gene expression was calculated by the comparative *C*t (threshold cycle) method using the 7500 Fast System Sequence Detection Software (Applied Biosystems). For COMT mRNA expression in normal and RCC clear cell tissues, TissueScan™ Kidney Cancer cDNA Arrays (OriGene Techologies) were subjected to quantitative polymerase chain reaction (Q-PCR) analysis. For investigation of gene expression profiling of selected apoptosis-related genes, the Human Apoptosis RT^2 Profiler PCR Array (SABiosciences, Frederick, MD) was used as per manufacturer's instructions. In brief, total RNA extracted from Caki-1/pCMV6 and Caki-1/COMT cells after 4-OHE_2 (50 μM) treatment for 48 h was converted into cDNA by using the RT^2 First Strand Kit. Q-PCR analysis was performed with RT^2 Real-Time™ SYBR Green/Rox PCR master mix. Gene expression levels were determined by using the data analyzer template provided by SABiosciences.

Western blot analysis

Whole-cell extracts were prepared using radioimmunoprecipitation assay buffer (Thermo Scientific, Rockford, IL) containing protease inhibitor cocktail (Roche Diagnostics). Total protein (40 μg) was loaded onto 12% Bis–Tris Gels with 3-(*N*-morpholino)propanesulfonic acid buffer and separated by a NuPAGE elecrophoresis system (Invitrogen). Protein was transferred to Invitrolon™ polyvinylidene difluoride and immunoblotting was carried out according to standard protocols. The antibodies used for immunoblotting were rabbit polyclonal antibody raised against COMT (Sigma–Aldrich, St Louis, MO) and GADD45α (Cell Signaling Technology, Danvers, MA), and mouse monoclonal antibody against GAPDH (Santa Cruz Biotechnology, Santa Cruz, CA) was used to confirm equal loading.

Immunohistochemistry

Immunostaining was performed on a formalin-fixed paraffin-embedded kidney cancer tissue array (US Biomax, Rockville, MD) using the ImmunoCruz Staining System (Santa Cruz Biotechnology). Briefly, the sections were deparaffinized in xylene and rehydrated in graded alcohols, followed by rinsing in dH_2O. After antigen retrieval by microwaving, endogenous peroxidase activity was quenched with H_2O_2 and rinsed with washing buffer. Normal goat serum was applied to minimize non-specific binding. The sections were incubated overnight at 4°C with rabbit polyclonal anti-COMT antibody (Sigma Life Sciences, St Louis, MO) diluted 1:250 in serum block followed by biotinylated anti-rabbit antibody. After incubation with horseradish peroxidase–streptavidin complex, Ultravision Plus DAB Plus Substrate System (Labvision, Fremont, CA) was added as chromogen followed by counterstaining with hematoxylin. Staining intensity of each tissue section was visually evaluated under an Olympus BX60 microscope equipped with Spot Advanced software (Diagnostic Instruments, Sterling Heights, MI) and was ranked on an overall scale from 0 to 3; with 0 indicating the absence of staining; 1, weak staining; 2, moderate staining and 3, strong staining. Assessment of the staining pattern was adapted and modified from the study of Fisher *et al.* (23). In brief, the presence or absence of staining, depth of color and the number of cells showing a positive reaction were recorded. Dark cytoplasmic staining that is easily visible with a low power objective and involves >50% of cells was considered as strong, focal dark staining areas (<50% of cells) or moderate cytoplasmic staining of >50% of cells was classified as moderate and focal moderate staining in <50% of cells or pale cytoplasmic staining in any proportion of cells not easily seen under a low power was categorized as weak.

Cell proliferation assay

Cells were plated in triplicate in 96-well microplates at a density of 5×10^3 cells per well in phenol red-free media supplemented with 10% charcoal-stripped fetal bovine serum (Invitrogen). The cells were then treated with 50 μM of either 4-OHE_2 or 4-ME. At the desired time point, the number of viable cells was determined by adding 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium-based CellTiter 96 AQueous One Solution Reagent (Promega, Madison, WI) to each well and measuring the absorbance at 490 nm on SPECTRA MAX 190 plate reader (Molecular Devices, Sunnyvale, CA). Results were expressed as the percentage of optical density, assuming that the absorbance of control cells was 100%. The working concentration of 4-OHE_2 and 4-ME was selected based on concentration–response studies (Supplementary Figure 1 is available at *Carcinogenesis* Online). In brief, cells were treated with 1, 10, 20, 50 and 100 μM of 4-OHE_2 or 2, 10, 20, 30 and 50 μM of 4-ME and cell proliferation was determined by 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium assay. As a result, 50 μM was chosen for both since it showed the most significant growth inhibitory effect on cells.

Apoptosis assay

Apoptosis was analyzed with an Annexin V-fluorescein isothiocyanate (FITC)/7-amino-actinomycin D staining system obtained from BD Biosciences (San Diego, CA). Briefly, cells were harvested and resuspended in binding buffer at a concentration of 1×10^6 cells/ml. For each assay, 1×10^5 cells were incubated with 5 μl of Annexin V-FITC and 20 μl of 7-amino-actinomycin D in the dark for 15 min at room temperature. After adding 400 μl of 1× binding buffer, samples were analyzed within an hour by a Cell Lab Quanta™ SC MPL (Beckman Coulter, Fullerton, CA).

Small interfering RNA transfection

When a cell density of 30–50% confluence was reached, small interfering RNA (siRNA) duplexes specific for human GADD45α or universal scrambled negative control (OriGene Techologies) was transfected using Lipofectamine 2000 Transfection Reagent (Invitrogen) as described by the manufacturer's instructions. Target specificity and knockdown efficiency were evaluated by both real-time PCR and western blotting with three different sets of siRNA duplexes at different concentrations. The siRNA duplex at a concentration of 20 nM showing the most efficient GADD45α knock down was selected and used for further experiments. Cells treated with Lipofectamine 2000 Transfection Reagent alone were included as a mock control for each experiment.

Statistical analysis

Values are presented as the mean ± standard error of mean based on results obtained from at least three independent experiments. Statistical significance

was evaluated by conducting a two-tailed unpaired Student's *t*-test using GraphPad PRISM Software. $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$, n.s, non-significant.

Results

COMT expression is downregulated in RCC tissues and cell lines

Given that a polymorphic variant of COMT, with a potential to alter its expression, is a risk factor for RCC (22), we initially examined whether COMT levels are changed in RCC tissues. Q-PCR studies showed significantly decreased expression of COMT mRNA in clear cell RCC compared with normal renal cells (Figure 1A). As previously reported (24), immunohistochemical staining of COMT protein was predominantly observed in proximal convoluted tubules in the juxtamedullary cortex (Figure 1B, left). Although we are unable to distinguish COMT isoforms, most of the immunoreactive COMT was located in the cytoplasm, with only occasional cell nuclei showing positive staining in normal renal cells (Figure 1B, left). In contrast, weak or no expression of COMT protein was observed in clear cell RCC tissues with average staining scores of 0.37 ± 0.16 (versus 2.63 ± 0.13 in normal tissues) (Figure 1B, right). The relative COMT expression levels in RCC cell lines were also compared with the immortalized kidney tubule epithelial cell line HK-2. Consistent with the observation in tissues, both COMT mRNA and protein levels were lower in RCC cell lines than in HK-2 cells (Figure 1C and D, respectively). The COMT protein occurs as two distinct forms, in the cytoplasm as a soluble protein and in association with membranes, mainly in the rough endoplasmic reticulum as a membrane-bound form (25). The human soluble (S)-COMT protein contains 221 amino acids and MB-COMT protein has 50 additional amino acids residues. Thus, their molecular weights are 25 and 30 kDa, respectively (25). Although both forms of COMT are expressed in equal amounts in human kidney (25), MB-COMT is predominantly expressed in both HK-2 and RCC cell lines (Figure 1D). These results showing lower COMT expression in cancer tissue and cells suggest that COMT may play a suppressive role in RCC tumorigenesis.

COMT overexpression enhances 4-OHE$_2$-mediated growth inhibition of RCC cells

Reduced expression of COMT in RCC tissues and cell lines led us to examine whether increased levels of COMT affects the growth of RCC. Since the majority of COMT is present as the MB form in RCC cell lines, we transiently transfected a plasmid containing human MB-COMT into Caki-1 cells, which has low level of endogenous COMT, and assessed the growth rate of the cells. However, there were no changes in proliferation, apoptosis and the cell cycle by transient upregulation of COMT in Caki-1 cells (data not shown).

Since COMT catalyzes the *O*-methylation of 2- or 4-OHE$_2$ and produces 2- or 4-ME, respectively (12), it may affect the growth of RCC in the presence of its metabolic substrates. Indeed, production of 2-ME by COMT expression inhibits the 17β-estradiol-induced proliferation of human uterine leiomyoma cells (21). However, a few studies have investigated the effect of 4-OHE$_2$ metabolism and 4-ME on cancer cell growth (26,27). Therefore, we explored the growth-inhibitory effect of 4-OHE$_2$ on COMT overexpressing RCC cells. To investigate this possibility, we first established Caki-1 and ACHN cell lines stably overexpressing MB-COMT. As shown in Figure 2A, a significant amount of exogenous protein was detected by western blotting in COMT-transfected RCC cell lines (Caki-1/COMT and ACHN/COMT) but not in control vector-transfected cells (Caki-1/pCMV6 and ACHN/pCMV6). Next, we treated the stable COMT-expressing cell lines with 4-OHE$_2$ and examined cell proliferation. Overexpression of COMT significantly enhanced the level of growth inhibition that occurs in response to 4-OHE$_2$ in

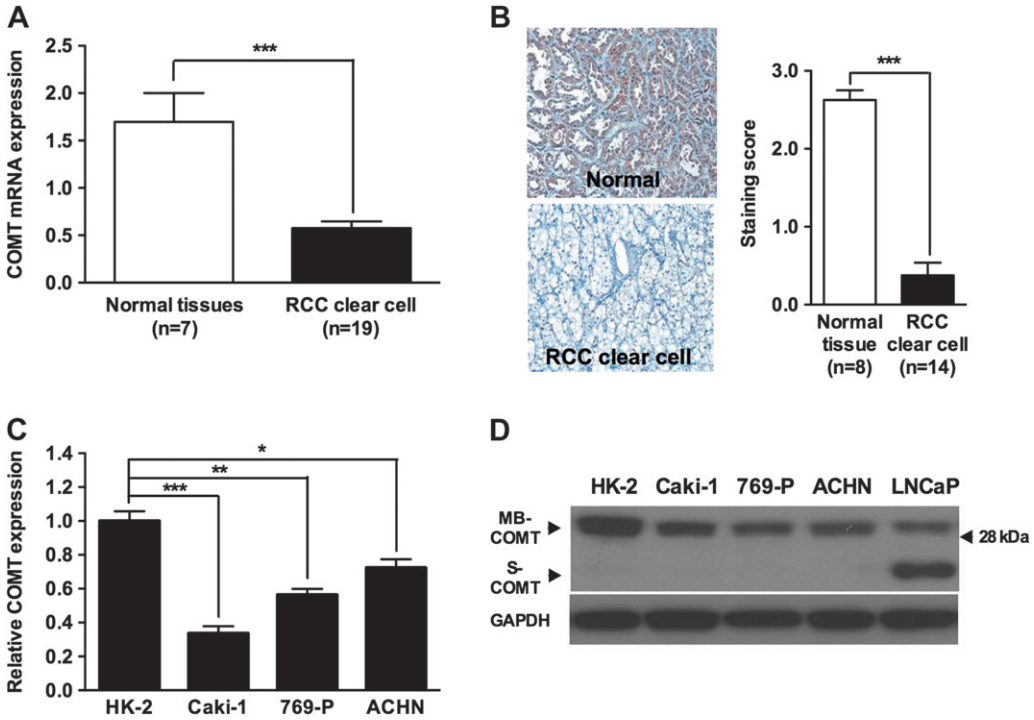

Fig. 1. Reduced COMT expression in RCC tissues and cells. (**A**) COMT mRNA expression in normal kidney and RCC clear cell tissues. Q-PCR was performed with TissueScan^{TM} Kidney Cancer cDNA Arrays. COMT signal was normalized to β-actin expression. (**B**) Immunohistochemical staining of COMT protein expression. Kidney cancer tissue array was immunostained with COMT antibody and counterstained with hematoxylin. Left, representative images showing immunoreactive COMT in normal and RCC clear cell tissues (magnification: ×200). Right, summary of COMT immunostaining score. Staining intensity was assessed as described under 'Materials and methods'. (**C**) Relative expression of COMT mRNA in RCC cell lines. Total RNA was extracted from the indicated cell lines and subjected to quantitative reverse transcription–PCR analysis. COMT signals were normalized to GAPDH. Data are presented as fold increase relative to COMT expression in HK-2 cells. (**D**) A representative immunoblot displaying COMT expression in RCC cell lines and LNCaP cells. LNCaP cells were used as a positive control to detect the two isoforms of COMT. MB-COMT: membrane-bound form of COMT; S-COMT: soluble form of COMT. Values are presented as the mean ± standard error of mean of three experiments. Asterisks denote statistically significant differences between the compared values: $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

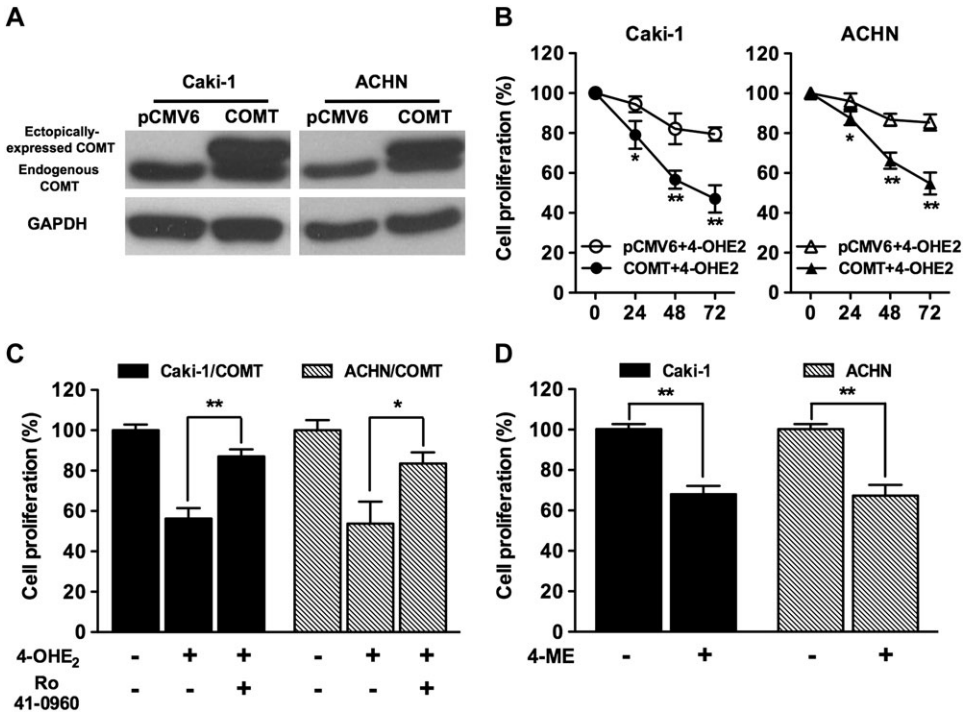

Fig. 2. Enhanced growth suppressive effect of COMT on RCC cells in the presence of 4-OHE_2. (**A**) Ectopic expression of COMT in RCC cell lines. COMT expression in stably transfected Caki-1 and ACHN cells with either COMT cDNA (Caki-1/COMT and ACHN/COMT) or empty vector (Caki-1/pCMV6 and ACHN/pCMV6) were determined using western blot analysis. GAPDH was used as a control for protein loading. (**B**) Proliferation of Caki-1/COMT and ACHN/COMT cells in response to 4-OHE_2. Cells were treated with 4-OHE_2 (50 μM) for the indicated times. (**C**) Effect of COMT inhibitor (Ro 41-0960) on the proliferation of Caki-1/COMT and ACHN/COMT cells in response to 4-OHE_2. Cells were exposed to 4-OHE_2 (50 μM) in the absence or presence of Ro 41-0960 (20 μM) for 72 h. (**D**) Influence of 4-ME on the proliferation of parental Caki-1 and ACHN cells. Cells were treated with 4-ME (50 μM) for 72 h. The number of viable cells were determined by reading the optical density produced with CellTiter® 96 AQueous Assay. Values are expressed as mean ± standard error of mean of three experiments. Asterisks denote statistically significant differences between the compared values: $^{*}P < 0.05$, $^{**}P < 0.01$.

a time-dependent manner (Figure 2B). Pretreatment of cells with COMT-specific inhibitor, Ro 41-0960 reversed the 4-OHE_2-mediated decrease of cell number suggesting this observation could be a COMT-specific effect (Figure 2C). As indicated in Figure 2D, 4-ME clearly caused growth inhibition of parental Caki-1 and ACHN cells. Thus, our data suggest that COMT inhibits the growth of RCC cells in the presence of 4-OHE_2 by producing 4-ME.

4-OHE_2 induces apoptotic cell death in COMT overexpressing RCC cells

Growth inhibition may result from the increase in cell cycle arrest and/or apoptotic cell death.

To further understand the growth-inhibitory effect of 4-OHE_2, we examined cell cycle distribution and mode of cell death by flow cytometric analysis. It was observed that 4-OHE_2 treatment leads to no change in cell cycle progression (data not shown). In contrast, the apoptotic cell fractions, which were stained with Annexin V-FITC only (early apoptotic) or both Annexin V-FITC and 7-amino-actinomycin D (late apoptotic), were significantly increased in 4-OHE_2 treated cells (Figure 3A and B). Ro 41-0960 reduced the 4-OHE_2-mediated apoptotic cell death (Figure 3B). These results suggest that 4-OHE_2-mediated growth inhibition results from increased apoptosis in COMT-expressing RCC cells.

4-OHE_2 upregulates GADD45α in COMT overexpressing RCC cells

To gain insight into the underlying mechanisms of the COMT-mediated growth inhibition of RCC cells, we examined changes in gene expression in Caki-1/COMT cells exposed to 4-OHE_2 using the Human Apoptosis RT^2 Profiler PCR Array containing Q-PCR primers of 84 known apoptosis-related genes. From the genes analyzed, the expression of eight genes was significantly increased and among them, GADD45α was greatest at 8.6-fold (Table I).

To verify the array data, we performed Q-PCR assay with a different probe than the one used in the PCR array. Consistently, a robust increase of GADD45α mRNA expression was detected in both Caki-1/COMT (7.8-fold) and ACHN/COMT cells (5.5-fold) (Figure 4A). Next, we explored whether the 4-OHE_2-induced elevation in GADD45α mRNA is associated with an increase in protein level. As shown by western blotting, GADD45α protein expression was significantly increased by exposure to 4-OHE_2 in both Caki-1/COMT and ACHN/COMT cells (Figure 4B). Furthermore, 4-ME promoted GADD45α protein expression in parental Caki-1 and ACHN cells (Figure 4C). Since GADD45α was the most dramatically regulated gene among those examined, we hypothesized that GADD45α may play a pivotal role in growth inhibition by 4-OHE_2 in COMT overexpressing RCC cells.

Inhibition of GADD45α prevents apoptotic cell death by 4-OHE_2 in COMT overexpressing RCC cells

To determine whether GADD45α is a key effector in the 4-OHE_2 metabolism-mediated downstream signaling pathways, we measured apoptotic cell death by exploiting GADD45α siRNA to selectively knock down GADD45α in RCC cells. We first confirmed the ability of siGADD45α duplexes to suppress endogenous GADD45α level. Administration of GADD45α siRNA to COMT overexpressing RCC cells decreased both basal mRNA (data not shown) and protein (Figure 5A) expression. In the presence of control siRNA, 16–21% of cells underwent apoptosis in response to 4-OHE_2. COMT overexpressing RCC cells treated with GADD45α siRNA, in contrast, were refractory to the proapoptotic activity of 4-OHE_2 (Figure 5A). Furthermore, we examined the possible role of GADD45α in the antiproliferative effect of 4-ME. GADD45α siRNA efficiently inhibited both basal GADD45α mRNA (data not shown) and protein (Figure 5B) expression in parental Caki-1 and ACHN cells. Silencing

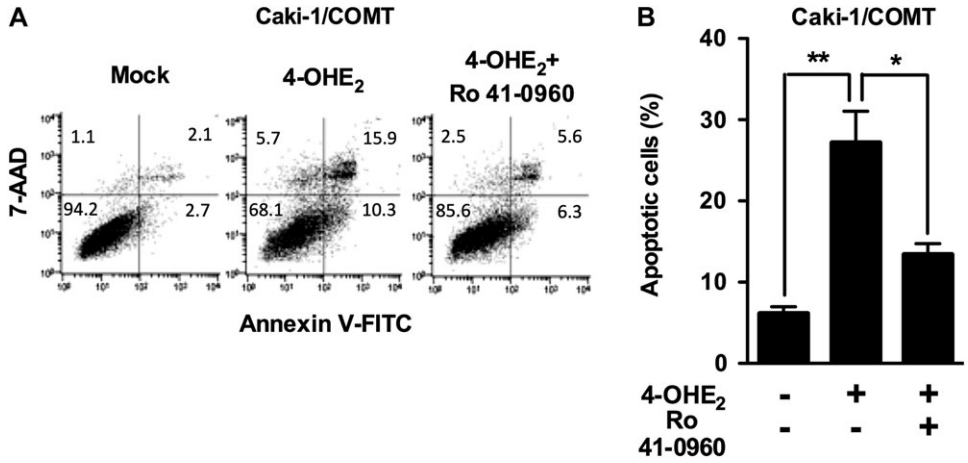

Fig. 3. 4-OHE_2-induced apoptotic cell death in COMT overexpressing RCC cells. Caki-1/COMT cells exposed to 4-OHE_2 (50 μM) with or without Ro 41-0960 (20 μM) for 72 h were subjected to flow cytometric analysis using double staining with Annexin V-FITC and 7-amino-actinomycin D (7-AAD). (**A**) A representative biparametric histogram showing cell population in early (bottom right quadrant) and late (top right quadrant) apoptotic and viable (bottom left quadrant) states. (**B**) Percent of total apoptotic cells. Values are expressed as mean ± standard error of mean of three experiments. Asterisks denote statistically significant differences between the compared values: $^{*}P < 0.05$, $^{**}P < 0.01$.

Table I. Summary of apoptosis-related genes significantly increased by 4-OHE_2 stimulation in Caki-1/COMT cells

Gene name[a]	Fold change[b]	*P* value
GADD45A	8.6	<0.001
LTA	4.2	<0.001
TRAF2	3.6	<0.05
TNFRSF10A	2.5	<0.01
BCL2A1	2.1	<0.01
AKT1	2.0	<0.001
BCL2L1	2.0	<0.001
CIDEB	2.0	<0.05

[a]Genes identified as increased ≥2-fold are listed.
[b]Fold change represents the ratio of signal in 4-OHE_2-treated Caki-1/COMT relative to Caki-1/pCMV6 cells for each primer set.

of the GADD45α gene by siRNA also prevented 4-ME-induced apoptosis (Figure 5B). These results demonstrate that GADD45α is an essential mediator of 4-OHE_2-induced apoptosis in COMT overexpressing RCC cells.

Discussion

It has been suggested that reduced enzymatic activity and altered expression of COMT are associated with the development and progression of various types of cancers. A polymorphic variant of COMT that may affect gene expression as a result of a change in RNA structure and thus, translation efficiency, could potentially lead to human RCC (22). Therefore, in the present study, we determined the expression level and functional significance of COMT in human RCC.

In the human kidney, most of the COMT expression is found in the cytoplasm of epithelial cells of convoluted tubules, in particular, of the juxtamedullary cortex region, which corresponds to findings in rat and hamster (24,28). A marked decrease of COMT expression in human RCC tissues and cells is also consistent with other studies observing no COMT immunoreactivity in hamster RCC (29,30). Although the mechanisms causing the decrease in COMT gene expression are not fully understood, there are three possibilities: First, estrogen regulates COMT expression and activity (30,31) and chronic exposure to estrogen at high doses leads to estrogen-associated cancer. Therefore, it is conceivable that high levels of estrogen in cancer patients may lead to COMT downregulation; second, there are many polymorphisms that have been identified in the COMT gene. COMT polymorphic variants in the coding region have been shown to result in either reduced enzyme activity or structural changes in mRNA, and as a result, cause altered protein expression (32,33). COMT polymorphism have been shown to correlate with RCC (22) and thus, variants may lead to lower expression in cancer; and third, aberrant methylation of the promoter region of genes is one of the major mechanisms for the loss of tumor suppressor gene function in human cancer (34). According to Sasaki *et al.* (35), COMT expression can be inactivated by CpG hypermethylation. Thus, analysis of the methylation status of the COMT promoter in RCC may give insight into the regulatory mechanism of COMT expression. However, despite these possibilities, the mechanisms behind the decreased levels of COMT in renal cancers are not clear and remain to be further defined.

In this study, we found that MB-COMT is predominantly expressed in RCC cell lines. This is also observed in a human cervical cancer (HeLa), malignant melanoma (SK-MEL-5), colon adenocarcinoma (Colo 320DM), neuroblastoma (IMR-32) and bone marrow (KG-1) cell lines (25). Whether this selective expression pattern is the case for RCC tissues is unknown since the antibody we used in the immunohistochemistry study detects both MB- and S-COMT, and high quality of antibodies which can specifically detect one of the isoforms are not commercially available.

It has been suggested that COMT may contribute to prevent the development of cancers in two ways. First, it reduces the level of 4-OHE_2, thereby lowering the potential for genomic damage through DNA adduct formation or through oxygen radicals arising from quinone–semiquinone redox cycling of catechol estrogen (15,16). Second, it converts 2-OHE_2 to 2-ME, which has antiproliferative, antiangiogenic and proapoptotic effects on multiple types of cancer (17). Unlike 2-ME, the biological function of 4-ME is largely unknown. Since it has little effect in experimental conditions where 2-ME is highly effective, 4-ME is believed to be primarily an end product of the COMT-mediated clearing mechanism of 4-OHE_2. However, in studies by Zhu *et al.* (36) and Dawling *et al.* (37), 4-ME serves as a substrate for 4-OHE_2 formation by demethylation catalyzed by CYP1B1. Moreover, Cheng *et al.* (38) showed that 4-ME can inhibit growth of human lung epithelial cells by inducing oxidative DNA damage via reactive oxygen species production.

Aside from the functions previously reported, the roles of 4-ME as an effective inhibitor of proliferation and an initiator of apoptosis in human RCC are newly discovered. Treatment of renal cancer cells with 4-ME fully mimicked the growth inhibition by GADD45α

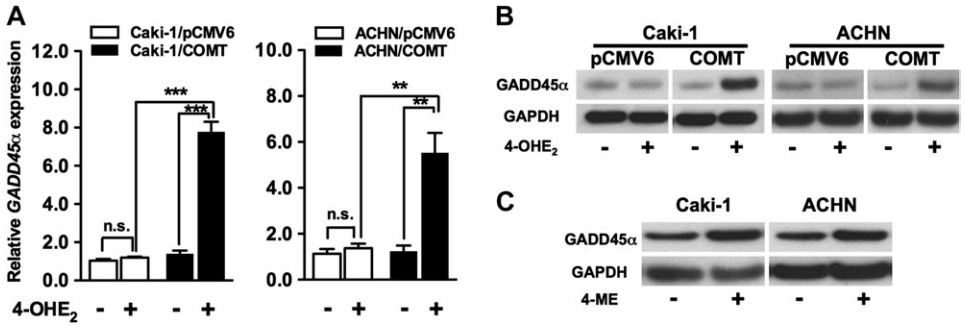

Fig. 4. Induction of GADD45α in COMT-expressing RCC cells by 4-OHE_2. (**A** and **B**) Induction of GADD45α mRNA (A) and protein (B) in COMT-expressing Caki-1 and ACHN cells after 4-OHE_2 exposure. Total mRNA or whole-cell lysates were extracted from pCMV6 or COMT expressing RCC cells treated with either vehicle or 4-OHE_2 (50 μM) for 48 h and subjected to quantitative reverse transcription–PCR (A) or western blot analysis (B), respectively. (**C**) Increased level of GADD45α by 4-ME in parental RCC cells. Whole-cell lysates were extracted from cells incubated with 4-ME (50 μM) for 48 h and subjected to western blot analysis. GAPDH was used as either a reference signal (A) or loading control (B and C). Values are expressed as the mean ± standard error of mean of three independent experiments. Asterisks denote statistically significant differences between the compared values: $^{**}P < 0.01$; $^{***}P < 0.001$; n.s, non-significant.

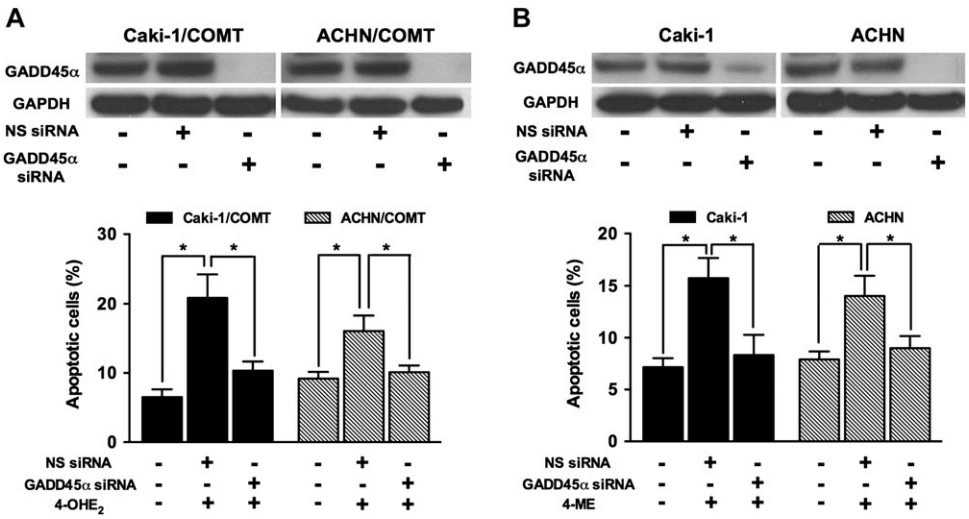

Fig. 5. Inhibition of 4-OHE_2-mediated apoptosis by GADD45α siRNA knock down. (**A** and **B**) Top, GADD45α siRNA suppress GADD45α protein expression. Caki-1/COMT and ACHN/COMT cells (A) or parental Caki-1 and ACHN cells (B) were transfected with non-specific (NS) control or GADD45α siRNA. After 48 h, cells were lysed and subjected to western blot analysis. GAPDH was used as loading control. Bottom, GADD45α siRNA protects against cell death caused by 4-OHE_2 or 4-ME. RCC cells were transfected with either NS control or GADD45α siRNA, maintained for 24 h and then treated with 4-OHE_2 (A) or 4-ME (B) as indicated. Early and late apoptotic cells were analyzed by flow cytometry. Values are expressed as mean ± standard error of mean of three independent experiments. Asterisks denote statistically significant differences between values compared: $^{*}P < 0.05$.

upregulation which were mediated by 4-OHE_2 in COMT overexpressing cells. In contrast to our results, no inhibitory effect of 4-ME on proliferation of endometrial, breast and cervical cancer cells has been reported (26,27). This discrepancy may arise from the different concentrations of 4-ME used for experiments. Kato *et al.* and Gong *et al.* measured cell viability at concentrations of 2 and 20 μM, respectively, whereas we used 50 μM based on the results with different dosages and indeed, cytotoxicity was observed at 30 μM of 4-ME in RCC cells (Supplementary Figure 1 is available at *Carcinogenesis* Online). With respect to 2-ME, the sensitivity of RCC cells is also different from that of endometrial cancer cells. While 5 μM 2-ME was capable of reducing viability of endometrial cancer cells by 30–35% (26,27), 70–75% reduction was observed in RCC cells (Chang,I. and Tanaka,Y, unpublished results). These data suggest that the growth-inhibitory effect of 4-ME might be cell-type specific or require higher concentrations in RCC cells compared with endometrial cancer cells.

In summary, our study have shown that COMT expression is decreased in human RCC and that ectopic COMT expression in response to 4-OHE_2 showed *in vitro* tumor suppressive abilities by affecting proliferation and apoptosis. A further study showed that the antiproliferative effect of COMT is due to 4-ME production and the resultant induction of GADD45α. Our study demonstrates the importance of COMT in the prevention of RCC development and provides support for COMT as a promising biomarker candidate and gene therapy targets for treating human RCC.

Supplementary material

Supplementary Figure 1 can be found at http://carcin.oxfordjournals.org/.

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