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RESEARCH ARTICLE

Expression profile of CYP1A1 and CYP1B1 enzymes in endometrial tumors

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Abstract The cytochrome P450 CYP1A1 and CYP1B1 enzymes are phase I extrahepatic enzymes involved in the activation of pro-carcinogenic compounds to carcinogenic metabolites. Although differential overexpression of CYP1A1 and CYP1B1 has been documented at the messenger RNA (mRNA) and protein level, studies that have examined CYP1 expression by enzyme activity assays are limited. In the current study, the expression of CYP1A1 and CYP1B1 was investigated in a panel of human tumors of endometrial origin by quantitative reverse transcriptase PCR (qRT-PCR), Western blotting, and enzyme activity assays. The data revealed that approximately 36 % (5/14) and 43 % (6/14) of the endometrial tumors overexpressed CYP1A1 and CYP1B1 mRNA, whereas in 57 % of the endometrial tumors, CYP1 mRNA levels were downregulated. The mean mRNA levels of CYP1B1 and CYP1A1 in endometrial tumors did not show a significant difference compared to normal tissues ($p>0.05$). Western blotting confirmed the qRT-PCR results and CYP1A1 and CYP1B1 proteins were shown to be downregulated in 7/14 (50 %) of the tumors and overexpressed in 4/14 (29 %) of the tumors. As regards to enzyme activity, 21 % (3/14) of the endometrial samples revealed elevated CYP1 activity levels across the tumor counterparts. Overall, the data suggest a putative downregulation of CYP1A1 and CYP1B1 expression in endometrial tumors, whereas overexpression of active CYP1 enzymes in 21 % of the tumors highlights the potential use of the latter enzymes as chemotherapeutic targets in endometrial cancer.

Keywords CYP1A1 · CYP1B1 · Cancer · Metabolism · Tumor expression

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Introduction

Endometrial cancer is a gynecological malignancy that is encountered at a high frequency worldwide. In Europe, endometrial cancer cases present with high frequencies with approximately 64,000 new cases annually and 15,000 morbidities each year [1, 2]. The treatment for endometrial cancer generally consists of surgery and chemotherapy. The chemotherapeutic drugs used for endometrial cancer include the DNA cross-linker, cisplatin, doxorubicin, and paclitaxel. Chemotherapy using the above mentioned compounds often results in unwanted side effects, notably bone marrow suppression and nephrotoxicity.

Cytochrome P450 CYP1 enzymes constitute the first family of CYPs and consist of three members, CYP1A1, CYP1B1, and CYP1A2. CYP1A1 and CYP1B1 are mainly extrahepatic enzymes that catalyze the oxidation of procarcinogens to carcinogenic reactive intermediates [3]. Consequently, the expression of CYP1A1 and CYP1B1 is an important contributor to carcinogenesis. The function of CYP1A1 and CYP1B1 is not limited to the metabolism of

drugs and carcinogens, as recent evidence suggests that CYP1 enzymes participate in the metabolism of important lipid mediators, such as arachidonic acid, to biologically active conversion products that are critical to the regulation of vascular tone, cardiac function, and acute inflammation [4]. In addition, CYP1A1 has recently been shown to affect cancer metastasis via catenin signaling and cancer proliferation via AMPK signalling [5–7]. Thus, constitutive overexpression of CYP1 enzymes in tumors does not necessarily promote cancer progression via the activation of pro-carcinogens, as the latter enzymes possess unique biological functions in addition to their xenobiotic-metabolizing roles.

The differential expression of CYP1A1 and CYP1B1 in various tumor types, compared to that in normal tissue, has been demonstrated by several studies, thus highlighting the potential use of the two CYP1 isoforms in cancer prognosis [8–11]. In addition, selective overexpression of CYP1A1 and CYP1B1 may be utilized to target specific tumor types by the activation of non-toxic prodrugs that are selectively metabolized to cytotoxic products [12, 13]. Thus, CYP1A1 and CYP1B1 play essential roles in cancer therapeutics, as well as in carcinogenesis.

While there is extensive evidence on the expression profile of CYP1A1 and CYP1B1, in terms of messenger RNA (mRNA) and protein levels, studies that have examined CYP1 enzyme activity are limited. Previously, we demonstrated that CYP1 enzyme activity and CYP1 mRNA is overexpressed in bladder and colon tumors. In the present study, we sought to examine the expression profile of CYP1A1 and CYP1B1 in human tumors of endometrial origin. Our observations indicate that CYP1A1 and CYP1B1 are possibly downregulated in endometrial tumors.

Materials and methods

Chemicals

4′-Methoxy-3′,5,7-trihydroxyflavone was purchased from Extrasyntheze (Genay, France) and 4′,3′,5,7-tetrahydroxyflavone from Sigma Aldrich (Dorset, UK). The C18 column for 4′-methoxy-3′,5,7-trihydroxyflavone and 3′,4′,5,7-tetrahydroxyflavone separation was purchased from Phenomenex (Cheshire, UK). The complementary DNA (cDNA) synthesis kit was purchased from Takara (Osaka, Japan). Polyclonal antibodies for CYP1A1 and CYP1B1 were from Millipore (AB 1258, MA, US) and Acris Chemicals (AP21718PU-N), respectively. Monoclonal antibody for β-actin was from Sigma (Poole, UK).

Patients and tumor specimens

Paired tumor and normal tissue samples from a series of 14 patients with primary endometrial cancer were acquired following routine pathological examination. The clinicopathological characteristics are shown in Table 1. Tumors were categorized as invasive (T2–T3) or non-invasive (T1). Patients were diagnosed with cancer from medical examination, medical records, and biopsy results. The 1973 WHO grading system was used in this study to classify the tumor grade of each sample acquired from patients with bladder and colon cancer.

Written informed consent forms were obtained for all patients from whom specimens were collected. The study protocol and consent procedure was approved by the Ethics Committee of the University of Crete.

Tissue specimens were collected during surgery from the tumor, placed in Eppendorf tubes, and stored at −80 °C until further use. A control sample was collected from the surrounding tissue area for each tumor specimen that was free of neoplastic infiltration.

RNA extraction and quantitative reverse transcriptase PCR

Total RNA was extracted from tumor tissues using TRIzol reagent as desribed previously [14]. Tissue fragments were homogenized in 1 ml TRIzol, mixed with 200 μl chloroform, and centrifuged for 15 min at 13,000 rpm. The top layer containing the RNA was mixed with 500 μl of ice-cold isopropanol and further centrifuged at 13,000 rpm for 10 min. The resulting pellet was washed once with 75 % ice-cold EtOH and resuspended in 40 μl of DEPC-treated water. The concentration of total RNA was estimated by UV/Vis spectrophotometry.

cDNA was synthesized from total RNA using a Takara RT kit according to the manufacturer's instructions. One microgram of RNA was mixed with H_2O and oligo(dT) primers, pre-heated for 5 min at 25 °C, and finally incubated at 43 °C for 1 h in the presence of DNTPs, reverse trancriptase, and reaction buffer containing $MgCl_2$ (5 mM).

Table 1 Demographic parameters of the subjects used in the study

Subjects	14
Age (mean)	63.7
Age (range)	46–72
Tumor type (endometrial)	14
Stage (T1)	3
Stage (T2)	9
Stage (T3)	2
Grade (I)	6
Grade (II)	3
Grade (III)	5

Reverse transcriptase PCR (RT-PCR) was carried out as described previously [14, 16]. cDNA of 0.5 μl was incubated with 8.3 μl of H_2O, 1.2 μl primers, and 10 μl SyBr master mix in a total volume of 20 μl per reaction. mRNA-specific primers for CYP1A1 and CYP1B1 were designed using Primer Express software 2.0 and validated in our previous study [14]. The primer sequences were as follows: CYP1A1 forward, CACCATCCCCCACAGCAC, and reverse, ACAA AGACACAACGCCCCTT; and CYP1B1 forward, GCTG CAGTGGCTGCTCCT, and reverse, CCCACGACCTGATC CAATTCT. The samples were subjected to 40 cycles of amplification with a denaturation step occurring at 95 °C (1 min), annealing step occurring at 60 °C (30 s), and chain elongation step occurring at 72 °C (30 s). Quantification was achieved by the use of a standard curve for *CYP1A1* and *CYP1B1* and normalization of the corresponding transcripts by the ΔΔCt method and the use of the housekeeping gene, *GAPDH* (forward primer, GCCCAATACGACCAAATCC, and reverse primer, AGCCACATCGCTCAGACA).

Western blotting

Protein samples from tissues were extracted using Trizol as described previously [14]. Protease inhibitor cocktail and DL-dithiothreitol (DTT, 1 mM) were added to each sample that was finally centrifuged at 13,000 rpm at 4 °C for 10 min. Protein concentration was estimated at 0.7 mg/ml for each sample and the protein lysate was mixed with sample buffer at 1:1 ratio. Samples were heated at 100 °C for 5 min and then loaded on an acrylamide gel. Electrophoresis was carried out for 1 h at 120 V and the proteins were transferred by wet blotting techniques to a PVDF membrane. The membrane was incubated in 5 % milk/0.05 % Tris-buffered saline with Tween 20 (TBST) at room temperature for 1 h by continuous shaking and further with primary antibody diluted in 1 % milk/0.05 % TBST at 4 °C overnight. The membrane was washed three times with 0.05 % TBST and incubated with secondary antibody against HRP diluted in 5 % milk/0.05 % TBST at room temperature for 1.5 h. The membrane was finally exposed with ECL reagents and the protein profile developed on a film. Antibodies for CYP1A1 and CYP1B1 were used at 1:500 dilutions, whereas antibody for β-actin was at 1:2,000 dilution. Secondary antibodies were used at 1:2,000 dilutions.

Microsome preparation

Microsomal pellets were prepared by differential ultracentrifugation [15]. Tissue samples were mixed with ice-cold phosphate buffer (10 mM PO_4) containing 20 % glycerol, protease inhibitors, and 1.15 % (*w*/*v*) KCl and homogenized using a mechanical homogenizer in 30-s bursts. The homogenate was centrifuged for at 9,000×*g* (30 min) at 4 °C, and the resulting supernatant was centrifuged for 60 min at 100,000×*g* at 4 °C in a Beckman 120 TLX ultracentrifuge. The microsomal pellet was washed once with 400 μl of 10 mM Tris-EDTA buffer (pH 7.4) containing 0.25 M sucrose and further centrifuged for 60 min at 100,000×*g* at 4 °C. The microsomal pellet was finally resuspended in 10 mM PO_4 containing 20 % glycerol and stored at −80 °C until further use. The protein concentration of each microsomal sample was estimated using the Bradford assay, and the determination of the P450 content of each sample was carried out using the reduced carbon monoxide spectrum.

CYP1 enzyme assays

CYP1 enzyme assays were performed using 4′-methoxy-3′,5,7-trihydroxyflavone (diosmetin) as a substrate, in the presence of NADPH (5 mM), $MgCl_2$ (0.5 mM), and phosphate buffer (20 mM, pH 7.4). Microsomal protein derived from tissues was used at a final concentration of 1 mg/ml. The samples possessed equal amounts of P450. Each reaction (100 μl) was initiated with the addition of microsomal protein carried out for 15 min and terminated by the addition of an equal volume of 1 % acetic acid in methanol. The samples were centrifuged at 3,500×*g* for 20 min at 4 °C and the supernatants analyzed by HPLC (Perkin Elmer 200, Wellesley, USA) or mass spectrometry (Shimadzu 2010 EV, Milton Keynes, UK).

HPLC and mass spectrometry analysis

The methodology used has been published in our previous studies [14, 16]. A Luna 5-μm C_{18} 4.6×150-mm column (Phenomenex, Cheshire, UK) was used, with a mobile phase flow rate of 1 ml/min, at a temperature of 37 °C. The mobile phase consisted of solvent A (1 % acetonitrile and 0.5 % acetic acid in water) and solvent B (4 % acetonitrile and 0.5 % acetic acid in methanol). The following gradient program was used: 60 % solvent A and 40 % solvent B at time 0, and 10 % solvent A and 90 % solvent B after 10 min. Final conditions were held for 1 min before returning to initial solvent conditions. Calibration curves for diosmetin and 4′,3′,5,7-tetrahydroxyflavone (luteolin) were performed at each run, using the following concentrations of compound standard: 10, 8, 6, 4, 2, 1, and 0.1 μM. Detection was performed at 360 nm.

Mass spectrometric analysis was run at positive/negative ionization (APCI) and 1.5-kV voltage of the detector, while retaining the initial LC parameter setup, in terms of solvents and gradient.

Statistical analysis

Significant differences between mean mRNA and activity levels in tumors and normal samples were investigated using

paired *t* test and Wilcoxon ranks test. The Mann–Whitney *U* test was employed to examine associations between CYP1A1/CYP1B1 expression and tumor stage. The level of significance was set at the 95 % confidence interval ($p<0.05$). The analysis was performed using SSPS software (version 15.0.1) and Microsoft Excel.

Results

Determination of CYP1A1 and CYP1B1 mRNA levels

By using mRNA-specific primers for CYP1A1 and CYP1B1 detection, RT-PCR was employed to access the levels of mRNA expression corresponding to the two genes. Dissociation curve analysis proved that no by-products were produced during the PCR reaction and that a single product was obtained for each gene (Fig. 1b). Levels of expression were quantified using a standard curve based on the amplification exponential curve corresponding to each PCR reaction (Fig. 1b). All of the 14 matched tumor and normal endometrial tissue pairs analyzed expressed detectable levels of CYP1B1 and CYP1A1 mRNA (Fig. 2). CYP1A1 and CYP1B1 mRNA showed a statistically significant upregulation across the tumor counterparts in 5/14 and 6/14 pairs, respectively, as determined by qPCR (Fig. 2). This corresponds to an approximate 36 and 43 % overexpression of CYP1A1 and CYP1B1 mRNA, respectively, in the tumor tissues. Eight out of 14 (57 %) endometrial tumor tissues revealed a significant downregulation in CYP1B1 and CYP1A1 mRNA expression, respectively, whereas in patient number 6, the expression levels of CYP1A1 mRNA between tumor and normal tissue did not reveal a significant change.

When mean expression levels of CYP1A1 and CYP1B1 mRNA were compared in the entire panel of endometrial tissues, the analysis indicated no significant differences between the normal and tumor counterparts, although (Fig. 3) the expression levels of CYP1B1 mRNA appeared to be lower in the endometrial tumors as opposed to those in normal tissues.

Determination of CYP1A1 and CYP1B1 protein levels

To further confirm our findings at the translational level, we employed Western blotting to investigate the expression of CYP1A1 and CYP1B1 proteins in the endometrial samples. The antibodies for CYP1A1 and CYP1B1 were polyclonal and purchased from Millipore and Acris, respectively. The analysis showed similar but not identical results to those obtained by the RT-PCR technique. CYP1A1 and CYP1B1 were overexpressed in 4/14

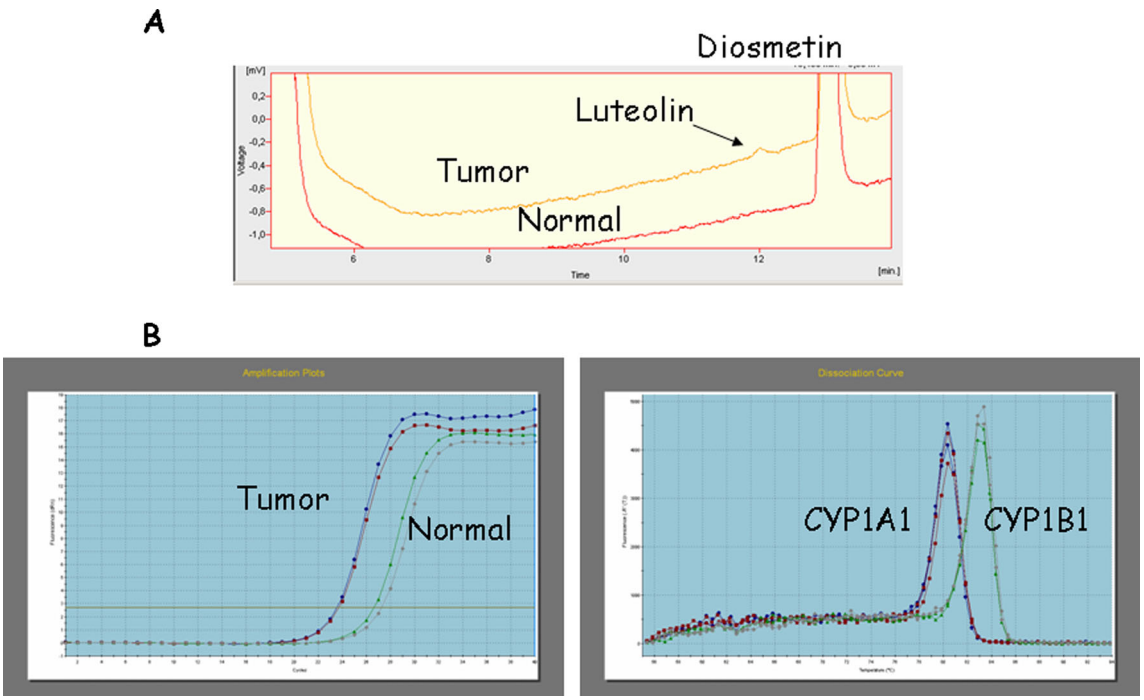

Fig. 1 Detection of CYP1 enzyme expression by qRT-PCR and enzyme activity assays. **a** Diosmetin (4′-methoxy-3′,5,7-trihydroxyflavone)-demethylase assay was used to detect CYP1 activity as published in our recent study [14]. Enzyme activity was calculated by the amount of luteolin (4′,3′,5,7-tetrahydroxyflavone) formed per time per protein concentration. Representative HPLC trace showing the identification of diosmetin and luteolin by reverse-phase HPLC following incubation of diosmetin with endometrial tumor or normal microsomes. **b** Determination of CYP1 mRNA levels was performed using mRNA-specific primers as described previously [14]. Representative trace depicting dissociation curves corresponding to the CYP1A1 and CYP1B1 gene and amplification of plots derived from tumor and normal tissue samples

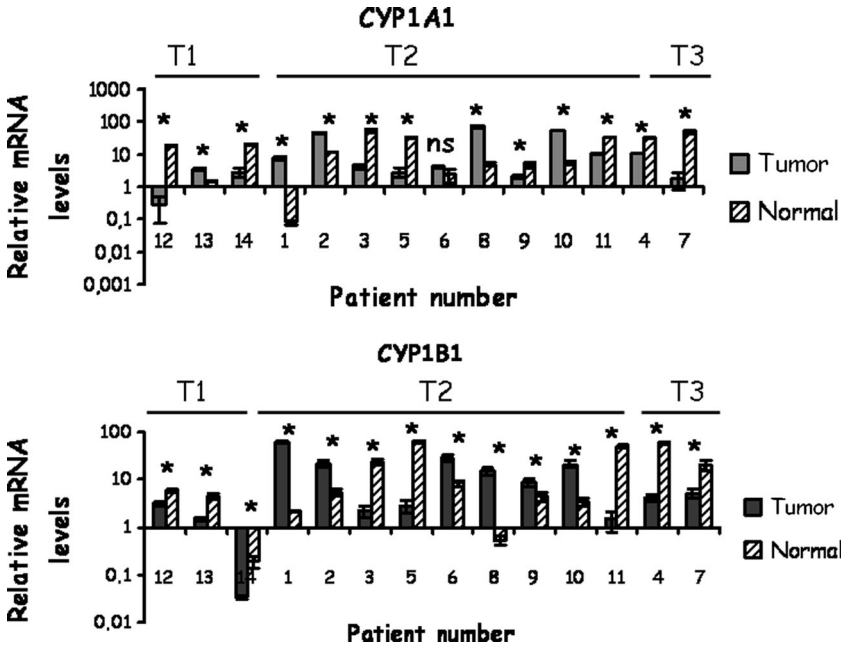

Fig. 2 Expression profiling of CYP1A1 and CYP1B1 mRNA expression in endometrial samples. qRT-PCR analysis of CYP1B1 and CYP1A1 in 14 matched normal and tumor pairs derived from endometrial tissue. Each *bar* represents an average of triplicate reactions. The numbers in the *x*-axis correspond to patient numbers. *T1*, *T2*, and *T3* represent the different tumor stages according to the TNM classification. *Ns* not statistically significant, *asterisk* statistically different $p<0.05$

endometrial samples that were derived from patient numbers 1, 2, 8, and 10 (Fig. 4a, b), as opposed to 5/14 and 6/14 samples where CYP1A1 and CYP1B1 were overexpressed at the mRNA level. Three out of 14 samples showed no significant difference in the protein levels of CYP1B1 or CYP1A1 whereas the remaining samples revealed downregulation of CYP1A1 or CYP1B1 protein with respect to the tumor counterpart (Fig. 4a, b). Correlation analysis revealed that the overexpression of CYP1A1 and CYP1B1 mRNA correlated with the overexpression of CYP1A1 and CYP1B1 proteins with coefficient values of 0.89 and 0.95, respectively (Fig. 4c).

Determination of CYP1 activity levels

The expression of CYP1 enzymes was determined by an activity assay, as described in our recent study based on

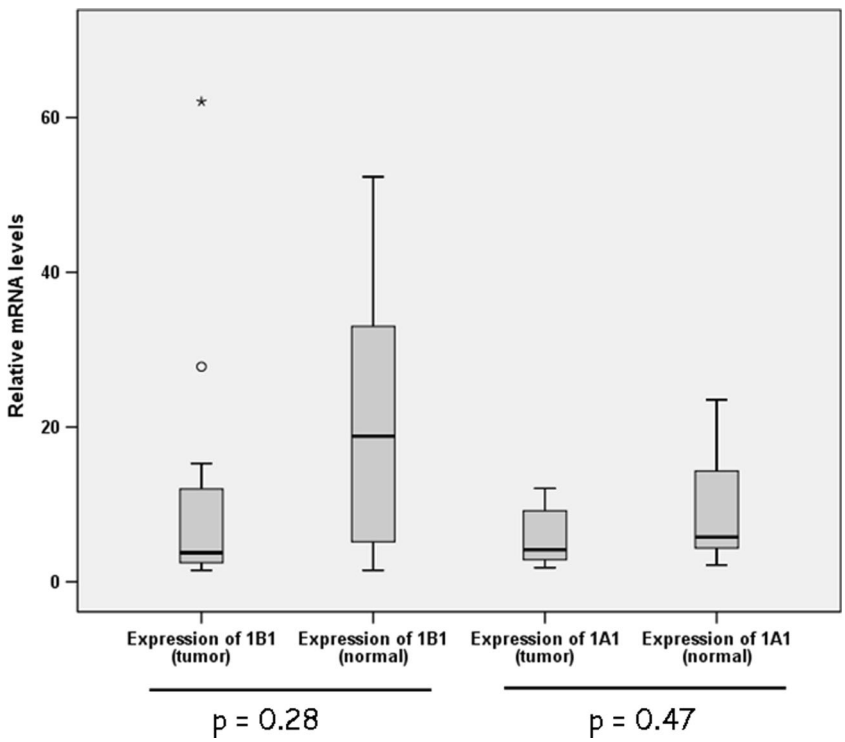

Fig. 3 Mean mRNA levels of CYP1A1 and CYP1B1 transcripts in human tumors. *Box plots* indicate the mean±SD for endometrial tumor and normal samples. Statistical analysis was conducted using paired *t* test and Wilcoxon ranks test. No significant differences were obtained for endometrial ($n=14$) versus normal ($p>0.05$ for CYP1A1 and CYP1B1) samples

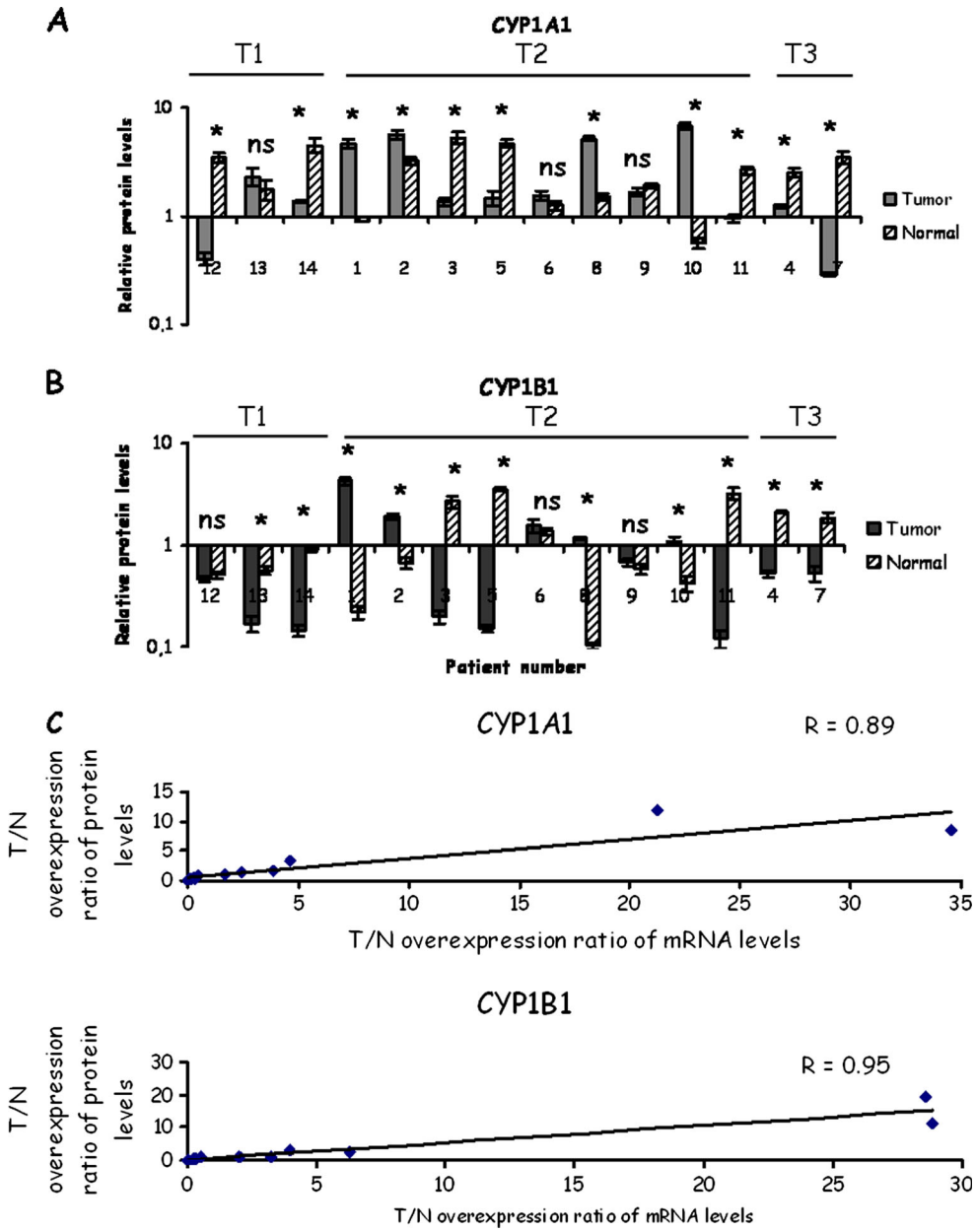

Fig. 4 Expression profiling of CYP1A1 and CYP1B1 protein expression in endometrial samples. Western blot analysis of **a** CYP1A1 and **b** CYP1B1 in 14 matched normal and tumor samples derived from endometrial tissue. The *numbers* in the *x*-axis correspond to patient numbers. *T1*, *T2*, and *T3* represent the different tumor stages according to the TNM classification. *Ns* not statistically significant, *asterisk* statistically different $p<0.05$. **c** Correlation of tumor to normal overexpression ratio of mRNA and protein levels using linear regression analysis. Correlation coefficients are indicated with *R* value

the demethylation of diosmetin [14]. The metabolism of diosmetin by CYP1A1 and CYP1B1 yields the product 3′,4′,5,7-tetrahydroxyflavone or luteolin (Fig. 1a). CYP1 activity in tumors was estimated by the amount of product formed per time per protein concentration (Fig. 5). Ten out of 14 endometrial samples did not express active CYP1 enzymes, at sufficient levels that could be detected by the assay used (data not shown). Activity levels were significantly higher in 3 out of 14 (21 %) endometrial tumors, whereas patient number 10 did not reveal a significant difference between the tumor and normal counterpart (Fig. 5b). The mean CYP1 activity in endometrial tumor samples ($n=4$) did not differ greatly from that found in the corresponding healthy tissues, although there was a tendency toward significance ($p=0.107$) (Fig. 5a).

Association between CYP1 enzyme expression and tumor pathology

The mRNA expression of the CYP1 genes was further investigated relative to the stage of the tumors. No statistically significant differences were obtained in the expression of each gene among the tumor groups T1/T2/T3 in endometrial tissues (data not shown).

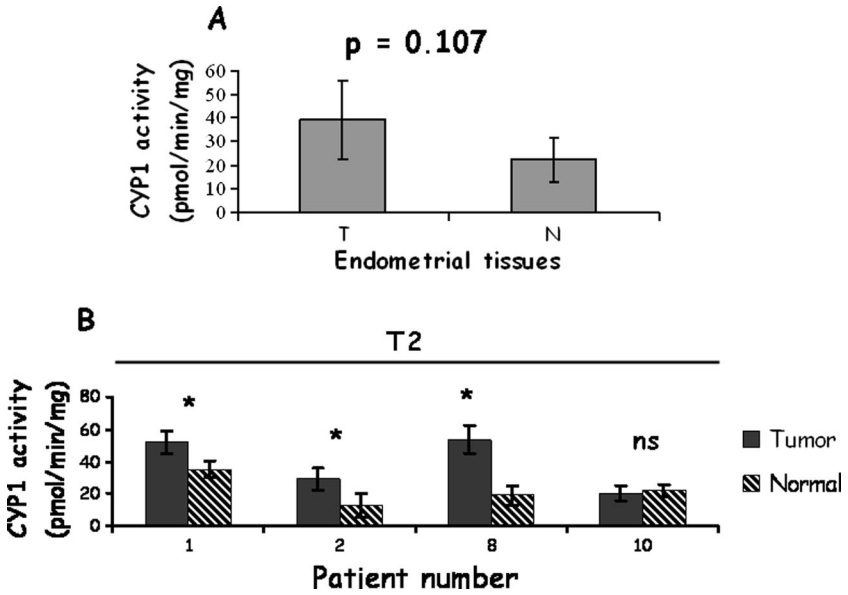

Fig. 5 Determination of CYP1 expression by enzyme activity assay. The *x*-axis corresponds to patient numbers, while the *y*-axis to CYP1 activity levels. Activity was calculated from the production of the metabolite, 3′,4′,5,7-tetrahydroxyflavone, per time per amount of microsomal protein. Each *bar* represents an average of triplicate reactions. **a** Mean CYP1 activity levels in human endometrial tumors (n=4). **b** CYP1 activity in individual endometrial samples. *ns* not statistically significant, *asterisk* statistically different $p<0.05$. *Bar charts* indicate mean±SD for endometrial (n=4) tumor and normal samples. Statistical analysis was conducted using paired t test and Wilcoxon ranks test. Significant differences between tumor and normal parts were obtained for endometrial samples that corresponded to patients 1, 2, and 8 ($p<0.05$)

Discussion

In the current study, an expression analysis of CYP1A1 and CYP1B1 enzymes in human tumors of endometrial origin was undertaken. Our results indicated that CYP1B1 and CYP1A1 mRNA expression was downregulated in 57 % of endometrial tumors, whereas CYP1A1 and CYP1B1 protein expression was downregulated in 50 % of endometrial tumors. The mean mRNA levels of CYP1A1 and CYP1B1 revealed no significant difference between normal and tumor tissues, although the data were suggestive of a putative downregulation of CYP1B1 mRNA expression in endometrial tumors (Fig. 3). With respect to CYP1 activity, only 3 out of 14 (21 %) endometrial samples presented with higher CYP1 activity compared to normal tissues.

Evidence regarding the expression profile of CYP1B1 in endometrial tumors is limited. A recent study that examined the expression of CYP1B1 by immunohistochemical techniques in 48 endometrial cancers and 24 controls showed approximately 50 % overexpression in the tumor samples [17]. In contrast, Hevir and colleagues detected lower levels of CYP1B1 by qRT-PCR and Western blot analysis in the cancerous endometrium compared to normal epithelium in 40 endometrial cancer specimens [18]. In the current study, no significant difference was obtained in the mean expression levels of CYP1 enzymes between tumor and normal endometrial samples, with respect to mRNA and activity levels. However, statistical analysis revealed a tendency toward significance (p=0.107) for mean CYP1 enzyme activity levels in endometrial tumors. It has been suggested that epigenetic modifications upon CYP1B1 promoters in tumor tissues may account for the downregulation of its expression, whereas induction of CYP1B1 by exogenous ligands of the Aryl hydrocarbon receptor may activate its transcription to active enzyme [19, 20].

To our knowledge, our study is the first that demonstrates active CYP1 enzyme expression in endometrial tumors. The group of Liehr and Ricci has demonstrated overexpression of CYP1B1 activity in mammary adenocarcinoma and human uterine myoma, compared to normal breast tissue and surrounding myometrial tissue, respectively, in two early reports [15, 21]. However, the activity assay used in the latter two studies was based on thin-layer chromatography, that is much less informative than high-pressure liquid chromatography. A more recent study performed by McFadyen et al. identified active CYP1B1 in renal cell carcinoma using an assay based on the metabolism of 7-ethoxyresorufin [22]. The results presented herein highlight an additional activity assay based on the demethylation of 4′-methoxy-3′,5,7-trihydroxyflavone (diosmetin). Activity of CYP1 enzymes can be detected by the production of the metabolite of 3′,4′,5,7-tetrahydroxyflavone (luteolin) and quantification via calibration curves containing authentic standards of both compounds.

Despite the relatively low percentages of CYP1 enzyme activity (21 %), our results indicate that the latter may be utilized as a therapeutic target for endometrial cancer via the

selective bioactivation of non-toxic prodrugs to potent cytotoxic metabolites. Examples of this type of targeted therapy include the benzimidazoles and duocarmycin compounds that are tested in phase I trials and preclinical models, respectively, for the treatment of breast and bladder cancer [23, 24]. Further studies are required to fully characterize which subtypes of endometrial tumors express active CYP1 enzymes and whether such a strategy can be employed as a successful form of treatment, although the results presented herein provide a first proof-of-principle concept of a potential cytochrome-P450-mediated anticancer drug therapy in endometrial cancer.

In conclusion, the current study demonstrates an expression analysis of cytochrome P450 CYP1A1 and CYP1B1 enzymes in a small sample size of endometrial tumors. The results are suggestive of a putative CYP1A1 and CYP1B1 downregulation in endometrial tumors, whereas the overexpression of active CYP1 enzymes was noted in 21 % of endometrial tumors, thus highlighting the potential use of CYP1 enzymes as therapeutic targets in endometrial cancer via the selective activation of non-cytotoxic prodrugs.

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Conflict of interest None

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