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Aldo-keto reductase activity after diethylhexyl phthalate exposure in eutopic and ectopic endometrial cells

Running title: Phthalate and AKRs in endometrial cells

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ABSTRACT

Objective: Endometriosis is a multifactorial gynaecological disease in reproductive-age women. Endometriotic tissue is characterized by high prostaglandin levels and progesterone resistance. Human aldo-keto reductases (AKRs) convert progesterone to a less potent

metabolite and cause progesterone resistance. Therefore, in this study, we evaluated whether diethylhexyl phthalate (DEHP) alters AKR expression in human ectopic and eutopic endometrium.

Study Design: We used microarrays and western blotting to study the effects of DEHP, and checked the presence of AKR in endometriosis patients by enzyme-linked immunosorbent assay (ELISA).

Results: Cultured human endometrial cells from normal endometrium of women without endometriosis (NE), eutopic endometrium from endometriosis patients (EE), and ectopic endometrium from endometriosis patients (EC) differed in genetic expression changes after DEHP treatment. DEHP upregulated AKR1C1, AKR1C2, AKR1C3, and AKR1B10 expression in EE, while EC showed continuously increased AKR1C3 expression before and after DEHP exposure. In western blot analysis, before and after DEHP exposure, the AKR1B10 protein band was detected in NE, EE, and EC, whereas the AKR1C3 band was detected only in EC. AKR1B10 and AKR1C3 expression levels in the blood of the enrolled patients were evaluated using ELISA. AKR1B10 expression did not differ between groups (without endometriosis [N = 13], 0.10 vs. with endometriosis [N = 20], 0.11; $P = 0.27$). AKR1C3 expression was significantly higher in the blood of endometriosis patients than in that of patients without endometriosis (without endometriosis, 9.1 vs. with endometriosis, 10.1; $P = 0.02$). Analysis according to menstrual period showed significantly increased AKR1C3 levels in patients with endometriosis only during the secretory phase and not the proliferative phase ($P < 0.05$).

Conclusion: DEHP induces AKR activity in the endometrium of endometriosis patients, and *AKR1C3* might influence the development of endometriosis.

Keywords: endometriosis, aldo-keto reductase

INTRODUCTION

Endometriosis is a gynaecological disease occurring in reproductive-age women. It is defined as the presence of endometrial tissues outside the uterine cavity. Endometriosis is not a malignant disease but is a chronic progressive disease, which causes pelvic pain, dysmenorrhea, and infertility. It is a multifactorial disease, the exact pathophysiology of which remains unclear.

Endometriotic tissue exhibits certain molecular characteristics that distinguish it from normal endometrium, e.g., abnormally increased proliferation, decreased apoptosis rate [1], increased angiogenesis, high prostaglandin levels [2], and progesterone resistance [3]. Progesterone resistance and decreased protective effects of progesterone are thought to constitute an important part of the pathogenesis of endometriosis. Beranic et al. [4] reported that progesterone resistance in endometriosis can arise from enhanced progesterone metabolism at the pre-receptor level. Human aldo-keto reductases (AKRs) catalyse these processes. Increased activity of AKR converts progesterone into the less potent metabolite 20α-hydroxyprogesterone, leading to a progesterone resistance effect [4]. AKR is also an important catalytic enzyme of prostaglandin biosynthesis. Increased AKR activity leads to an increase in prostaglandins, which is one of the important characteristics of endometriosis. Prostaglandins are mediators of the inflammatory reaction and stimulators of cell proliferation, migration, adhesion, and angiogenesis [2], which predominantly characterise ectopic endometriosis tissue.

Diethylhexyl phthalate (DEHP) is a widely-used phthalate and endocrine-disrupting

chemical (EDC), suspected to be a mediator of gynaecological disorders. In an earlier study [5], we assessed *AKR* gene expression in an endometrial cancer cell line (ECC-1) after DEHP treatment. DEHP treatment increased the gene expression of AKRs, stimulated prostaglandin production, and caused abnormal cell cycle in ECC-1 cells [6]. In this study, we hypothesised that the expression of *AKR* genes in human ectopic endometrium and eutopic endometrium might be altered by DEHP exposure. We aimed to assess the effects of DEHP and determine the level of AKR in the patients with endometriosis.

MATERIALS AND METHODS

Tissue collection and cell culture

Patients who visited Seoul St. Mary's Hospital for gynaecologic surgeries were enrolled for the present study. Endometrial tissues were collected from (i) uteruses with benign ovarian cysts but no endometriosis (NE: normal endometrium) (ii) uteruses of endometriosis patients (EE: eutopic endometrium), and (iii) ovarian endometrial cysts (EC: ectopic endometrium). Informed consent was obtained from each patient before surgery for the use of tissues for the present study. Approval for the use of endometrial tissue was granted by the Institutional Review Board of Seoul St. Mary's Hospital. The patients ranged in age from 20 to 43 years, with a mean age of 32 years, and all were in the proliferative phase.

Endometrial tissues were dissected from the uteruses and washed with Hank's balanced salt solution. The tissues were minced into small pieces and digested in 30 mg/mL collagenase IV and 4 mg DNase I at 37°C for 3 h. The endometrial cells were collected by centrifugation at 1,200 rpm for 3 min and washed 3 times with PBS. Isolated cells were cultured in 100-mm^2 culture dishes at 37°C under 5% CO_2 in a humidified atmosphere in phenol red-free Dulbecco's modified Eagle's medium/F12 (Gibco Life Technologies, Grand

Island, NY, USA) supplemented with 10% FBS, and 100 U/mL penicillin. The morphological characteristics were observed daily with an inverted microscope, and the medium was changed every 2 days. When the cells reached saturation, they were isolated with trypsin-EDTA.

Drug treatment

Each cell type was treated with 50 μM DEHP (Sigma-Aldrich, St. Louis, MO, USA) for 48 h.

RNA extraction and microarray

Total RNA was extracted from endometrial tissue by using TRIzol Reagent™ (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. Following homogenisation, 1 mL of solution was transferred to a 1.5-mL Eppendorf tube and centrifuged at 12,000 ×*g* for 10 min at 4°C to remove insoluble material. The supernatant containing RNA was collected, mixed with 0.2 mL chloroform, and centrifuged at 12,000 ×*g* for 15 min at 4°C. RNA in the aqueous phase was transferred into a new tube, precipitated by mixing 0.5 mL of isopropyl alcohol, and recovered by centrifuging the tube at 12,000 ×*g* for 10 min at 4°C. The RNA pellet was washed briefly in 1 mL of 75% ethanol and centrifuged at 7,500 ×*g* for 5 min at 4°C. Finally, the total RNA pellet was dissolved in nuclease-free water, and its quality and quantity were assessed by Agilent Bioanalyzer 2100 analysis. Gene expression was analysed with GeneChip® Human Genome U133 plus 2.0 arrays (Affymetrix, Santa Clara, CA, USA), which comprise over 45,000 probe sets representing approximately 38,500 well-characterised human genes. For each gene, 11 pairs of oligonucleotide probes were synthesised *in situ* on the arrays.

Biotinylated cRNAs were prepared from 250 ng total RNA according to the standard

Affymetrix protocol (Expression Analysis Technical Manual, 2001, Affymetrix). Following fragmentation, 15 μg of antisense RNA was hybridised for 16 h at 45°C on a GeneChip Human Genome Array. GeneChips were washed and stained in the Affymetrix Fluidics Station 450. GeneChips were scanned using the Affymetrix GeneChip Scanner 3000 7G. The data were analysed by Robust Multichip Analysis using Affymetrix default analysis settings and global scaling as the normalization method. The trimmed mean target intensity of each array was arbitrarily set to 100. The normalised and log-transformed intensity values were then analysed using GeneSpring GX12.5 (Agilent Technologies, CA, USA). Fold-change filters required that the genes be present in at least 200% of controls for upregulated genes and in fewer than 50% of controls for downregulated genes.

Quantitative real-time PCR

cDNAs were synthesised using 1 μg of total RNA according to the manufacturer's protocol (High-Capacity RNA-to-cDNA Kit; Applied Biosystems). Quantitative real-time PCR (qPCR) was performed in triplicate in 384-well plates. A 384-well high-throughput analysis was performed using the ABI Prism 7900 Sequence Detection System (PE Applied Biosystems, www.appliedbiosciences.com) and white-coloured 384-well plates (ABgene, Hamburg, Germany). The fluorescence emission from each sample was collected by a charge-coupled device-camera, and the quantitative data were analysed using the Sequence Detection System software (SDS version 2.2, PE Applied Biosystems). Reaction mixtures contained 10 pmol/μL of each primer and 2× SYBR Green PCR Master Mix (PE Applied Biosystems), which includes the HotStarTaq DNA polymerase in an optimised buffer, the dNTP mix (with dUTP additive), SYBR Green I fluorescent dye, and ROX dye as a passive reference. Each of the 384-well qPCR plates included serial dilutions (1, 1/2, 1/4, 1/8, and 1/16) of cDNA, which were used to generate relative standard curves for genes. All primers

(Table 1) were amplified using the same conditions. Thermal cycling conditions were as follows: 50°C for 2 min; 95°C for 10 min; and 40 cycles of 95°C for 30 s, 60°C for 30 s, and 72°C for 30 s. To exclude the presence of nonspecific products, melting curve analysis of the products was performed routinely after amplification by high-resolution data collection during an incremental temperature increase from 60°C to 95°C with a ramp rate of 0.21°C/s. We then converted qPCR cycle numbers to gene amounts (ng) on the basis of the equation. The qPCR analysis was performed on an Applied Biosystems Prism 7900 Sequence Detection System (PE Applied Biosystems).

Western blot analysis

Proteins were extracted from cultured cells. After DEHP treatment, NE, EE, and EC cells were lysed at 4°C for 30 min in the presence of a lysis buffer. The lysates were subsequently centrifuged at 12,000 ×g for 30 min at 4°C, and the supernatants were collected. Protein content in the supernatants was determined by the BCA assay. Each 30-µg aliquot of the protein extracted from cultured cells was separated by NuPAGE Novex 4–12% Bis-Tris gel (Invitrogen Life Technologies) under reducing conditions at 200 V for 50 min. Proteins were then electrophoretically transferred from gels to PVDF transfer membranes (Amersham Biosciences). The blots were exposed overnight to anti-AKR1B10 and anti-AKR1C3 rabbit polyclonal antibodies (Abcam, Bristol, UK). The membranes were incubated for 1 h with horseradish peroxidase-conjugated anti-mouse or anti-rabbit secondary antibodies (Santa Cruz Biotechnology, Inc. CA, USA) diluted at 1:1000 with blocking buffer. The antigen–antibody complexes were detected with the ECL chemiluminescence detection system (Amersham Biosciences). Membranes were visualised by exposure to X-OMAT film (Eastman Kodak Co., Rochester, NY, USA).

ELISA

Thirty-three patients who visited Seoul St. Mary's Hospital for gynaecologic surgery were enrolled. Twenty patients underwent surgery for endometriosis, and 13 patients underwent surgery for other reasons. Approval for the use of the endometrium was granted by the Institutional Review Board of Seoul St. Mary's Hospital. Whole blood (3 mL) was obtained during the proliferative phase and secretory phase from each patient after 8 h of fasting, in the out-patient department before surgery. A specific sandwich ELISA kit was used to quantify AKR1B10 and AKR1C3 in human plasma. The assay is specific for human RANTES and has no known cross-reactivity with other cytokines or chemokines. Aliquots of human plasma samples were each tested in triplicate at several dilutions and compared to reference standards of recombinant human AKR1B10 and AKR1C3. AKR1B10 and AKR1C3 concentrations were measured using an ELISA kit (MyBioSource, CA, USA) according to the manufacturer's protocol.

RESULTS

Microarray data

Microarray data analysis of endometrial cells from normal endometrium (NE) indicated that the expression of 231 genes changed significantly in cell ontology: 127 genes were upregulated, and 104 genes were downregulated (1.5-fold change) after DEHP treatment. In endometrial cells from endometriosis patients' eutopic endometrium (EE), 4,086 genes showed significant changes: 1,706 genes were upregulated and 2,380 genes were downregulated after DEHP treatment. In endometrial cells from endometriosis patients' ectopic endometrium (EC), 463 genes showed significant changes: 429 genes were

upregulated and 35 genes were downregulated after DEHP treatment. The gene expression levels of the treatment group and control group were compared, and statistical significance was set at a 1.5-fold difference in the degree of gene expression at a *P*-value of <0.05. We analysed 3 independent samples to determine the degree of RNA transcription. The expression and fold changes of *AKR* genes after DEHP treatment are shown in Table 1.

qPCR data

qPCR was performed using the fluorescent dye SYBR Green to assess the degree of mRNA expression of *AKR* genes (*AKR1B10*, *AKR1C1*, *AKR1C2*, *AKR1C3*) identified by performing microarray analysis of RNA extracts of 3 types of endometrial cells (NE, EE, and EC) treated with DEHP. The qPCR findings corroborated the microarray mRNA expression data (Figure 1). The PCR primers and probe sequences used for qPCR are listed in Table 2. In NE, AKR1B10 expression was significantly increased, which differed from microarray data. In EC, AKR1B10, AKR1C1, AKR1C2, and AKR1C3 showed no significant expression change after DEHP treatment, similar to the expression change pattern in the microarray data.

For checking the potential of AKR as a marker to detect endometriosis, relative quantification analysis of qPCR results based on the EC expression level was performed (Figure 2). Before DEHP treatment, *AKR1B10* and *AKR1C3* expression levels showed larger differences between NE and EE. We analysed AKR1B10 and AKR1C3 proteins by western blot analysis (Figure 3) and ELISA (Figure 4) for checking the actual protein level in cells.

Western blot analysis

Before and after DEHP exposure, the AKR1B10 protein band was detected in NE, EE, and EC, whereas the AKR1C3 protein band was detected only in EC (Figure 3).

ELISA

AKR1B10 and AKR1C3 expression levels were checked in the blood of the enrolled patients. (with endometriosis; N = 20, without endometriosis; N = 13). AKR1B10 expression did not differ between groups (without endometriosis, 0.10 vs. with endometriosis, 0.11; $P = 0.27$). AKR1C3 expression was significantly higher in the blood of endometriosis patients than in patients without endometriosis (without endometriosis, 9.1 vs. with endometriosis, 10.1; $P = 0.02$). Analysis according to menstrual period showed significantly increased AKR1C3 level in patients with endometriosis only during the secretory phase and not the proliferative phase (Figure 4). During the secretory phase, the mean serum AKR1C3 level was significantly higher in the patients with endometriosis than in those without endometriosis ($P < 0.05$).

COMMENT

In the present study, normal endometrial tissue did not show increased *AKR* gene expression after DEHP exposure, but eutopic endometrial tissue did. After the classical retrograde menstruation theory was put forth, some researchers reported that not all endometrial tissue resulting from retrograde menstruation forms ectopic endometriosis lesions [7,8], and only genetically altered endometrial cells get implanted outside the uterus [9]. However, what triggers such a genetic change has not been unveiled.

AKRs are involved in prostaglandin synthesis and progesterone resistance pathways, which are commonly noticed in endometriosis patients. The activity of AKRs increased after exposure to certain nutrients, drugs, toxins, or xenobiotics [10-12] because of its

detoxification activity [13-15]. Increased AKR superfamily activity after exposure to some environmental factors has a protective function in decreasing their toxic activity, but it sometimes acts like a pathogenic factor [16,17].

Eutopic endometrial tissue of endometriosis patients showed increased AKR activity after exposure to EDCs, and these changes might have triggered progesterone resistance and prostaglandin synthesis. These changes were not checked in normal endometrial tissue. These results imply that some triggering factors that cause an increase AKR levels might induce the onset of endometriosis. We surmise that the determining factors for endometriosis include not only the exposure level of EDCs but also the ability of *AKR* gene expression to change after EDC exposure. The increased serum AKR1C3 in patients with endometriosis in this study is thought to be a manifestation of toxin-induced gene expression changes.

DEHP is most commonly used as a plasticiser, and it is known to be associated with decreased oestradiol synthesis [18], decreased aromatase activity [19], and altered natural ovulation timing, resulting in anovulatory cycles and polycystic ovarian syndrome in females [20]. The daily exposure level of DEHP is reported to be 0.003–0.03 mg/kg/day (7.7–77 μM, MW 390.56 g/M) in USA [21]. The concentration of DEHP used in the present study was 50 μg/kg/day, which simulates the clinical situation.

Small population size was a limitation of this study. Therefore, a larger study on serum AKR1C3 level and its relationship with endometriosis is warranted. Nevertheless, we can conclude that DEHP induces AKR activity in the endometrium of endometriosis patients, and elevated AKR activity might play a role in the emergence of endometriosis.

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FIGURE LEGENDS

Figure 1. Gene expression changes after DEHP treatment obtained by microarray and qPCR assays. The qPCR data and microarray analysis data showed similar gene expression change patterns in 3 cell types. NE, endometrial cells from normal endometrium; EE, endometrial cells from endometriosis patients' eutopic endometrium; EC, endometrial cells from endometriosis patients' ectopic endometrium.

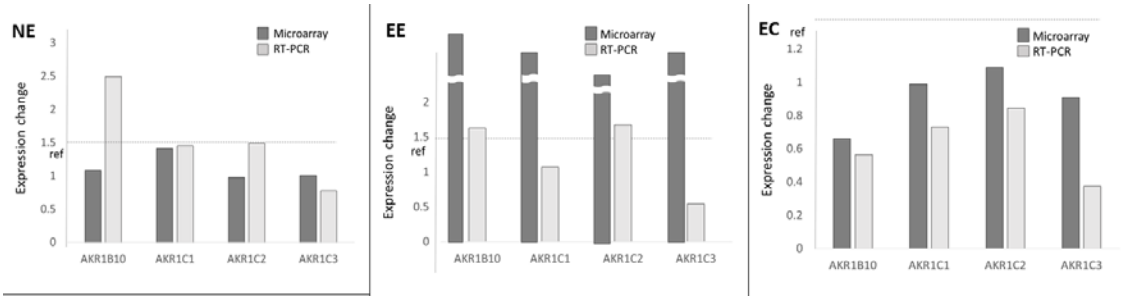

Figure 2. Relative quantification analysis of qPCR data. Gene expression quantification data of *AKR1B10* and *AKR1C3* showed a larger difference between NE and EE compared to EC. NE, endometrial cells from normal endometrium; EE, endometrial cells from endometriosis patients' eutopic endometrium; EC, endometrial cells from endometriosis patients' ectopic endometrium.

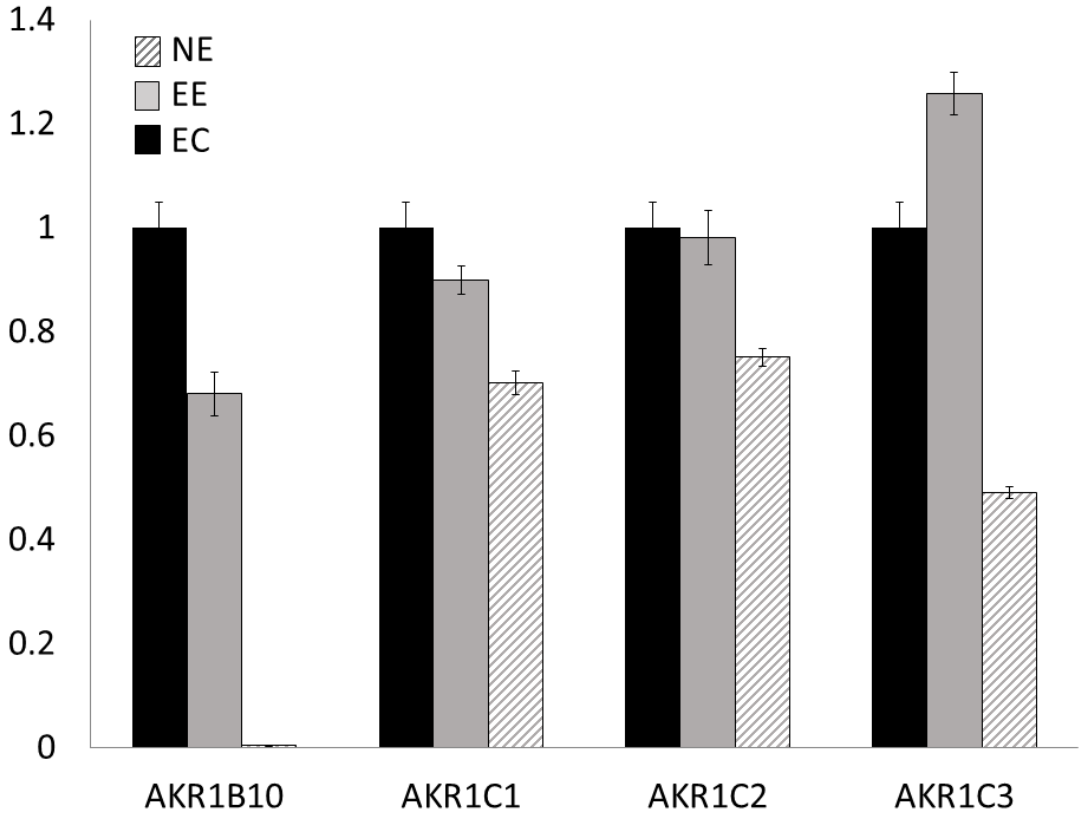

Figure 3. Western blot analysis of AKR1B10 and AKR1C3 in endometrial cells with or without DEHP treatment. NE, endometrial cells from normal endometrium; EE, endometrial cells from endometriosis patients' eutopic endometrium; EC, endometrial cells from endometriosis patients' ectopic endometrium.

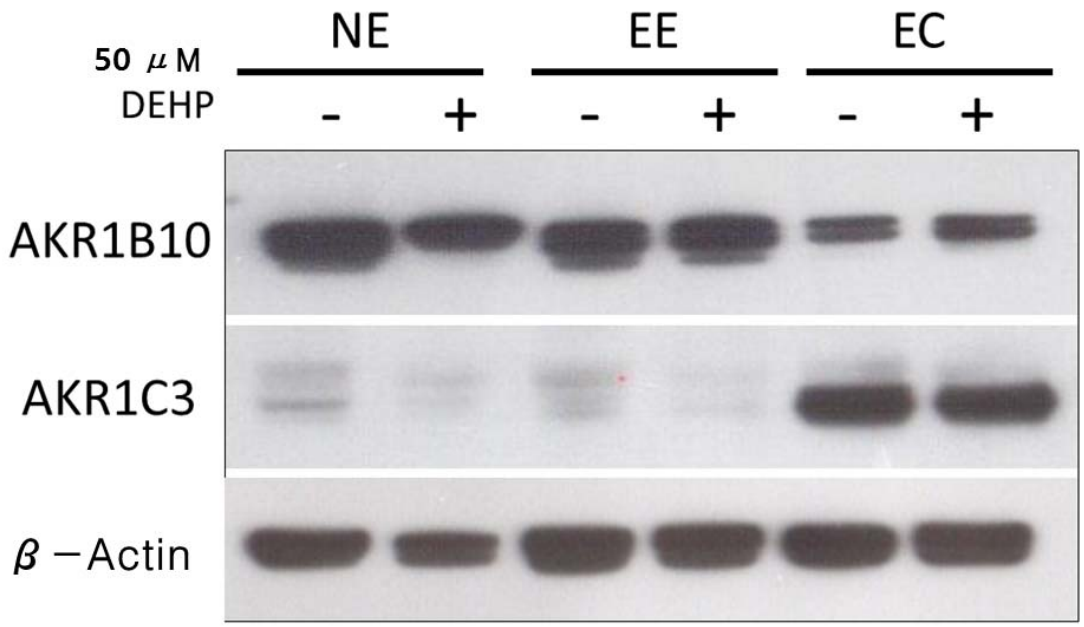

Figure 4. ELISA results of AKR1C3 in patients with or without endometriosis. During the secretory phase, the mean serum AKR1C3 level was significantly higher in patients with endometriosis than in those without endometriosis (†: $P < 0.05$).

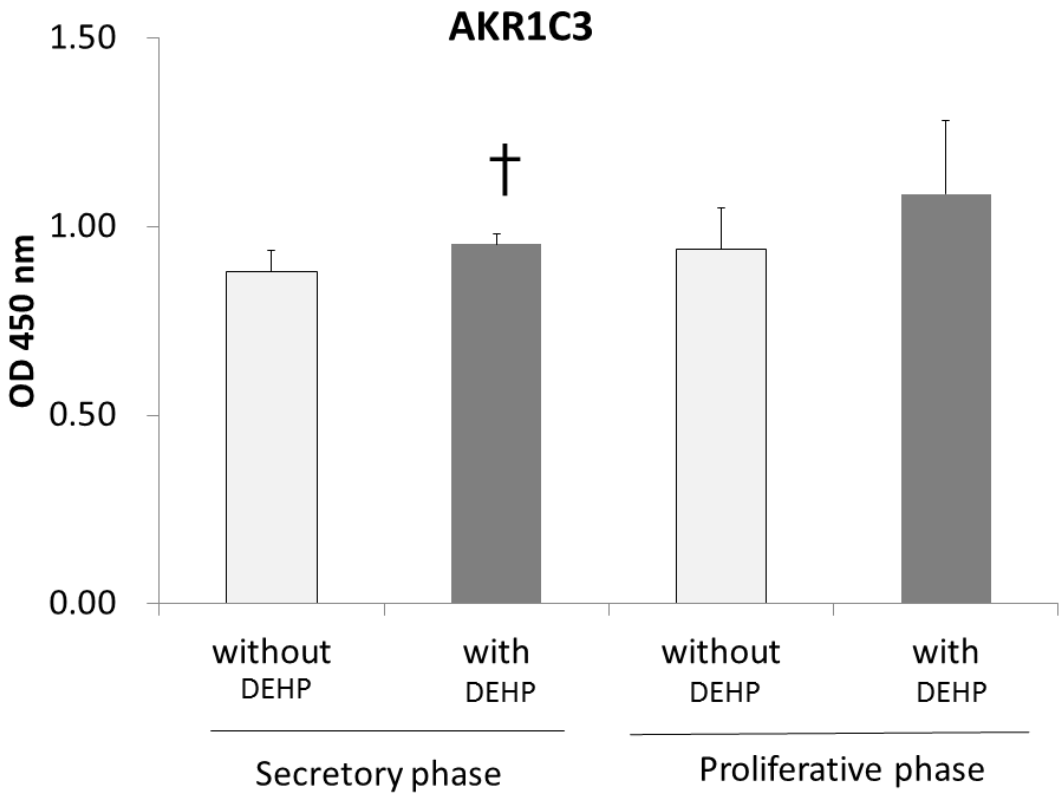

Table 1. Gene expression intensity of aldo-keto reductase of endometrial cells from endometriosis patients and control individuals after DEHP exposure.

GenBank accession no.	**Gene symbol**	**Gene name**	**Mean intensity (Cy5/Cy3)**		
			NE	EE	EC
NM_020299	AKR1B10	Aldo-keto reductase family 1,	1.08	**46.50**	0.66

		member B10			
NM_001353	AKR1C1	Aldo-keto reductase family 1, member C1	1.41	**23.31**	0.99
M33376	AKR1C2	Aldo-keto reductase family 1, member C2	0.98	**9.53**	1.09
AB018580	AKR1C3	Aldo-keto reductase family 1, member C3	1.01	**20.24**	0.91

Bold numbers indicate significant changes (1.5-fold change, $P < 0.05$). NE, endometrial cells from normal endometrium; EE, endometrial cells from endometriosis patients' eutopic endometrium; EC, endometrial cells from endometriosis patients' ectopic endometrium.

Table 2. Quantitative real-time PCR primer sequences.

GenBank accession no.	Gene symbol	Primer sequence (5′→3′)	
		Forward primer	Reverse primer
NM_020299	AKR1B10	AACGTGTTGCAATCCTCTCA	AACAGCACCTCGATTCTCGT
NM_001353	AKR1C1	TGGCTCTATGCTGGTGACTG	ATGGGCTTTGCTGTAGCTTG

M33376	AKR1C2	CAGGGCTCAAGTACAAGCCT	ACCCATGGTTCTTCTCGATG
AB018580	AKR1C3	ACGTCTCTATGCCGGTGACT	TTCTGGCCTATGGACTCAGC
	GAPDH	ATGGGGAAGGTGAAGGTCG	GGGGTCATTGATGGCAACAAT