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Original Article

Suppressed expression of type 2 3α/type 5 17β-hydroxysteroid dehydrogenase (AKR1C3) in endometrial hyperplasia and carcinoma

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Abstract: The diagnosis of endometrial hyperplasia and endometrial type adenocarcinoma arising within the uterine cavity has long been rested on morphologic criteria. Although distinction between normal endometrial epithelium from adenocarcinoma is usually straightforward, the separation between normal and hyperplastic endometrium, particularly those cases without atypia, can be a diagnostic challenge. The same is true in separation of hyperplastic endometrium with atypia from endometrial-type endometrial adenocarcinoma. Type 2 3α-/type 5 17β-hydroxysteroid dehydrogenase (HSD) (AKR1C3) is a multifunctional enzyme involved in androgen, estrogen, progesterone, and prostaglandin metabolism. Its expression has been shown in the epithelium of the renal tubules, urothelial epithelium, and endothelial cells in normal tissues as well as in prostatic adenocarcinoma. The proliferation and maintenance of endometrial epithelium is dependent on both estrogen and progesterone; and AKR1C3-mediated steroid metabolism may play a critical role in the maintenance of viable normal and abnormal endometrial epithelium. We studied the expression of AKR1C3 in 33 endometrial biopsy specimens including 13 cases of normal proliferative endometrium, 8 cases of hyperplastic endometrium with and without atypia, and 12 cases of primary endometrial adenocarcinoma of endometrial type. We demonstrated a uniform, diffuse, and strong expression of AKR1C3 in normal endometrial epithelium but not in endometrial stromal cells. In contrast, the expression of AKR1C3 is reduced in both hyperplastic and carcinomatous endometrial epithelium. These findings suggest that AKR1C3 may play important roles in the physiology of endometrial cells and that suppressed AKR1C3 expression may represent a feature that allows differentiation of hyperplastic and neoplastic endometrial epithelium from normal endometrial epithelium. However, reduced AKR1C3 expression cannot distinguish hyperplastic endometrium from endometrial adenocarcinoma of endometrial type. The biologic and pathological roles of AKR1C3 in endometrial epithelium require further investigation.

Keywords: Aldo-keto reductase, endomtrial cancer, estrogen, progesterone, prostaglandin

Introduction

Normal endometrial function requires an orchestrated interplay between different steroid hormones, including estrogen and progesterone [1]. Based on biochemical and clinical studies, the concentration of 17β-estradiol in endometrial carcinoma tissue is significantly higher than its concentration in normal endometrium [2] and excess or prolonged estrogen exposure unopposed by progesterone increases the risk of endometrial carcinoms [3, 4]. On the other hand, progesterone is absolutely necessary for maintaining the decidual phenotype before menstruation and during pregnancy through supporting endometrial cell survival [5]. There is increasing evidence that progestagen supplementation can antagonize estrogen-activated cell proliferation and protect against the development of endometrial cancer [4]. Enzymes

that are responsible for intratumoral steroid hormone biosynthesis and metabolism have been suggested to play cardinal roles in steroid-dependent epithelial neoplasm such as breast cancer [6]. However, the roles of steroid hormone metabolizing enzymes in endometrial carcinoma remain unclear.

The aldo-keto reductases (AKRs) comprise a functionally diverse 15 gene families [7]. Members of the AKR superfamily are generally monomeric (37 kD), cytosolic, and NAD(P)(H)-dependent oxidoreductases that share a common $(\alpha/\beta)_8$-barrel structural motif. This family of enzymes convert carbonyl groups to primary or secondary alcohols (www.med.upenn.eud/akr) [8]. Natural substrates for these enzymes include steroids, prostaglandins (PGs), and lipid aldehydes [9]. In humans, at least four AKR1C isoforms exist; they are known as AKR1C1 [20α (3α)-hydroxysteroid dehydrogenase (HSD)] [10], AKR1C2 (type 3 3α-HSD) [11, 12], AKR1C3 (type 2 3α/type 5 17β-HSD) [13, 14], and AKR1C4 (type 1 3α-HSD) [12].

AKR1C3 was originally cloned from human prostate [14] and placental cDNA libraries [15]. AKR1C3 has 3α-HSD, 17β -HSD, and 11-ketoprostaglandin reductase activities [16] which catalyze androgen and PG metabolism [11, 14, 16]. AKR1C3 also converts estrone (weak estrogen) to 17β-estrodiol (potent estrogen) and progesterone to 20α-hydroxyl-progesterone through reductive activity, and the reverse reactions through its oxidative activity [17]. As a result, AKR1C3 is capable of indirectly governing ligand access to various nuclear receptors, including androgen receptor (AR), estrogen receptor (ER), progesterone receptor (PR), and peroxisome proliferator-activated receptor (PPAR) [18], and regulating *trans*-activation activities of these nuclear receptors.

The presence of AKR1C3 has been demonstrated in both steroid-dependent and non-steroid-dependent cells including the Leydig cells [19], urothelial epithelium, epithelium of the renal tubules [20], and endometrial cells [21]. Deregulated expression of AKR1C3 has been demonstrated in multiple types of cancers, including breast cancer [22], lung cancer [23], prostate cancer [24-27], and Wilms' tumor [28]. In contrast to earlier reports suggesting that AKR1C3 expression is significantly elevated in endometrial hyperplasia and endometrial adenocarcinoma of endometrial type [21, 29], we demonstrated immunohistochemical evidence of reduced expressions of AKR1C3 in hyperplastic and carcinomatous endometrial epithelium as compared to proliferative phase endometrial epithelium.

Materials and Methods

Materials

Mouse anti-AKR1C3 monoclonal antibody was produced in our laboratory [30]. Biotinylated goat-anti mouse IgG antibody and horseradish peroxidase (HRP)-conjugated streptavidin were obtained from Vector Laboratories (Burlingame, CA). Stable diaminobenzidine tetrahydrochloride (DAB) and goat serum were purchased from Invitrogen (Carlsbad, CA). Hematoxylin and permount mounting media were obtained from Sigma-Aldrich (St. Louis, MO).

Human Tissues

Archival, formalin-fixed, paraffin-embedded non-hyperplastic/non-neoplastic, hyperplastic, and malignant human endometrium specimens were procured in the Departments of Pathology and Obstetrics and Gynecology at the University of Oklahoma Health Sciences Center. Human tissue specimens were obtained and processed with Institutional Review Board (IRB) approval. A total of 33 endometrial biopsy specimens, all from different patients, were obtained for this study. This consortium included 13 cases of control proliferative endometrium which is defined as proliferative endometrium without hyperplasia, neoplasia, or atrophy, 8 cases of hyperplastic endometrium with and without atypia, and 12 cases of primary endometrial adenocarcinoma of endometrial type. Out of the 8 cases of hyperplastic endometrium, atypia is present in 6 of the 8 cases. In the cases with adenocarcinoma, the cases ranged from International Federation of Gynecology and Obstetrics (FIGO) grade 2 to 3 and nuclear grade 2 to 3. The age of these patients ranged from 17 to 94 years old and all of the endometrial samples without evidence of hyperplasia, neoplasia, or atrophy (control endometrium) were obtained from women between 17 and 51 year of ages.

Immunohistochemistry of Tissue Sections

Immunohistochemistry of human tissue sections was performed as per our previously re-

ported procedures [25] in duplicates. Briefly, tissue sections cut about 4-6 μm were mounted and baked at 60 °C for 1 hr. Sections were deparaffinized with xylene and re-hydrated in graded ethanol followed by rinses with 0.1 M Tris-HCl (pH 7.6). Endogenous peroxidase activity was blocked by incubating the tissue sections with 1.6% H_2O_2 in methanol for 30 min. Antigen retrieval was performed with 0.01 M sodium citric acid buffer (pH 6.0) at 95 °C for 1 hr. Non-specific binding was blocked by incubating the tissue sections with 0.1 M Tris-HCl containing 10% goat serum for 2 hr. AKR1C3 was then detected by incubating the sections with mouse anti-AKR1C3 monoclonal antibody (clone NP6G6.A6) at a 1:200 dilution in the above blocking solution in a moist chamber at 4 °C overnight. Negative controls were performed in parallel in the absence of the primary antibody. After washes with 0.1 M Tris-HCl, the sections were treated with 1:400 dilution of biotinylated horse anti-mouse secondary antibody and incubated at room temperature for 2 hr. Following another rinses with 0.1 M Tris-HCl, antibody binding was detected by incubating the tissue sections with HRP-conjugated streptavidin at room temperature for 30 min. DAB-H_2O_2 substrate was then added to the slides and incubated at room temperature for an additional 4 min. Tissue sections were counter stained lightly with hematoxylin, dehydrated in graded alcohol, cleared in xylene, and mounted with Permount Mounting Media for visualization by light-microscopy.

Histological and Pathological Evaluation of Endometrium Specimens

The diagnoses were confirmed and the stained sections were evaluated independently by two pathologists (KMF and VZ) using a conventional light microscope. The percentage of positive cells within the entire population of epithelial cells were evaluated and assigned to one of the following categories: negative to positivity ≤ 5%, positivity >5% but ≤ 25%, positivity >25% but < 75%, and 100% positivity. The intensity of immunoreactivity was also evaluated for being weak, moderate, and strong for every case.

Results

Endometrium without Evidence of Hyperplasia, Neoplasia, or Atrophy (control endometrium)

A total of 13 biopsy specimens were studied. Of these specimens, 6 of them contained only unremarkable endometrium and findings in the remaining specimens include stromal breakdown (**Table 1**). Immunoreactivity was evaluated and demonstrated 100% immunoreactivity (**Table 1** and **Table 2**) in all of the epithelial cells (Figure 1). Stromal cells were consistently negative. Endothelial cells, as we have reported before [20, 25], were also strongly positive with both nuclear and cytoplasmic immunoreactivity. Strong immunoreactivity in the epithelium was noted in all of these specimens.

Hyperplastic Endometrium with and without Atypia

A total of 8 biopsy specimens of hyperplastic endometrium were studied including 3 specimens without atypia and 5 specimens with atypia (**Table 1**). When immunoreactivity was positive, it was present only in epithelial cells and endothelial cells. Stromal cells were consistently negative. Out of these specimens, only 1 specimen contained 100% immunoreactivity that was diagnosed as complex hyperplasia with atypia. A range of immunoreactivity was demonstrated in the remaining specimens (**Table 1**) from 10% to 75%; and an intensity of immunoreactivity ranged from weak to moderated (**Figure 1**). The immunoreactivity in all but one was weaker than the control endometrium. There was 1 specimen (12.5%) with 75% to 100% positive immunoreactivity, 4 specimens (50%) with 25% to 75% positive immunoreactivity, 3 specimens (37.5%) with 5% to 25% positive immunoreactivity, and no specimen under 5% immunoreactivity (**Table 2**). The percentage of positive cells did not correlate with whether atypia was present.

Endometrial Adenocarcinoma

A total of 12 biopsy specimens of primary endometrial adenocarcinoma arising from the endometrium were studied; and these specimens classified as FIGO grades 2 and 3, and nuclear grades 2 and 3 (**Table 1**). When immunoreactivity was positive, they were present only in epithelial cells and endothelial cells. Stromal cells were consistently negative. In 3 of these specimens (25%), 100% positive immunoreactivity was present in all tumor cells. In 4 of these specimens (33.3%), there was immunoreactivity in 25% to 75% of the tumor cells. In another 4 of these specimens (33.3%), there was immu-

Table 1. Percentage of positivity in proliferative, hypeplastic, and neoplastic endometrial tissue

Case	Age	Diagnosis	Percentage of Positive Cells & Immunoreactivity [1, 2]
Control			
1	36	Proliferative endometrium	☐☐☐*
2	37	Proliferative endometrium	☐☐☐*
3	28	Proliferative endometrium with stromal break down, polyp	☐☐☐*
4	32	Proliferative endometrium	☐☐☐*
5	28	Proliferative endometrium	☐☐☐*
6	36	Proliferative endometrium	☐☐☐*
7	29	Proliferative endometrium with stromal break down,	☐☐☐*
8	28	Disordered proliferative endometrium with focal stromal breakdown	☐☐☐*
9	31	Proliferative endometrium with focal early secretory phase	☐☐☐*
10	31	Proliferative endometrium and endometrial polyp	☐☐☐*
11	17	Normal Proliferative endometrium focal stromal breakdown	☐☐☐*
12	25	Proliferative endometrium with mild stromal breakdown	☐☐☐*
13	51	Proliferative endometrium	☐☐☐*
Hyperplastic			
1	51	Simple hyperplasia without atypia	☐
2	45	Endometrium with simple hyperplasia without atypia	☐
3	54	Complex hyperplasia with atypia	☐
4	41	Complex hyperplasia with atypia	☐☐
5	35	Simple hyperplasia without atypia	☐☐
6	60	Complex hyperplasia with atypia	☐☐
7	50	Complex hyperplasia with atypia	☐☐
8	46	Complex hyperplasia with atypia	☐☐☐
Neoplastic			
1	54	Endometrial adenocarcinoma, F2, N1	|
2	51	Endometrial adenocarcinoma, F3, N2	☐
3	46	Endometrial adenocarcinoma, F3, N3	☐
4	42	Endometrial adenocarcinoma, F2, N2	☐
5	62	Endometrial adenocarcinoma, F2, N2	☐
6	68	Endometrial adenocarcinoma, F3, N2	☐☐
7	76	Endometrial adenocarcinoma, F2 N2	☐☐
8	94	Endometrial adenocarcinoma, F2, N2	☐☐
9	61	Endometrial adenocarcinoma, F3, N2	☐☐
10	52	Endometrial adenocarcinoma, F2, N2	☐☐☐
11	36	Endometrial adenocarcinoma, F2, N2, focal squamous differentiation	☐☐☐
12	59	Endometrial adenocarcinoma, F2, N2	☐☐☐

[1] The percentage of positive cells are depicted as follows:
☐☐☐: >75-100% of cells are positive.
☐☐: >25-≤75% of cells are positive.
☐: >5-≤25 of cells are positive.
|: 5% or less positive cells

[2] The intensity of immunoreactivity is depicted as follow:
☐: Strong
☐: Moderate
☐: Weak

* Endometrial glands from endometrium without hyperplasia, neoplasia, or atrophy demonstrated 100% strong immunoreactivity in all samples.

noreactivity in 5% to 25% of the tumor cells. In one of the cases (8.3%), there was less than 5% positive immunoreactivty in tumor cells. (**Table 2**)

Table 2. Summary of immunoreactivities

Diagnosis	Percentage of positive cells	No. of cases
Proliferative endometrium without evidence of hyperplasia or neoplasia (13 cases)	>75% to 100%*	13 (100%)
	≤75%, >25%	0 (0%)
	≤25%, >5%	0 (0%)
	≤5%	0 (0%)
Endometrial hyperplasia with or without atypia (8 cases)	>75% to 100%	1 (12.5%)
	-≤75%, >25%	4 (50%)
	≤25%, >5	3 (37.5%)
	≤5%	0 (0%)
Endometrial adenocarcinoma, endometrial type (12 cases)	>75% to 100%	3 (25%)
	≤75%, >25%	4 (33.3%)
	≤25%, >5	4 (33.3%)
	≤5%	1 (8.3%)

* Endometrial glands from endometrium without hyperplasia, neoplasia, or atrophy demonstrated 100% strong immunoreactivity in all samples.

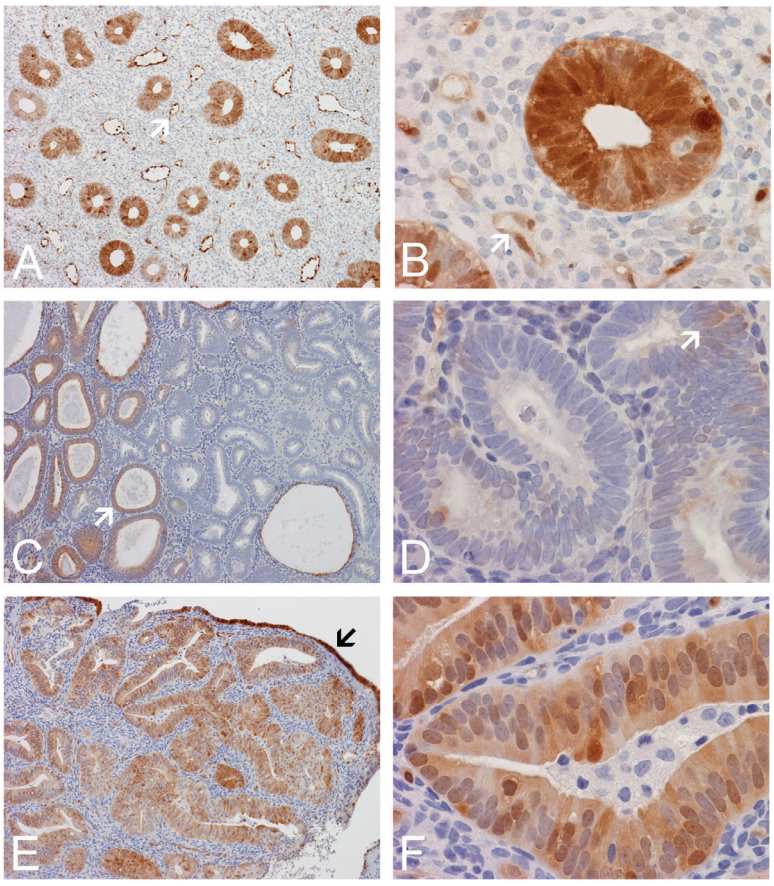

Figure 1: Expression of AKR1C3 in normal and hyperplastic endometrium. (A) Normal proliferative endometrial glands and blood vessels (arrow) are uniformly immunoreactive for AKR1C3 but endometrial stromal cells are negative. (B) Both nuclear and cytoplasmic immunoreactivity are noted in both endometrial glands and endothelial cells of blood vessels (arrow). (C) In this case of complex hyperplasia with atypia, only about 30% of the hyperplastic glands are immunoreactive for AKR1C3. Both positive (arrow) and negative areas are included here. (D) In some areas of this case, weak immunoreactivity limited to scant cells is demonstrated (arrow). (E) In this case of complex hyperplasia with atypia, there is immunoreactivity in practically in all of the hyperplastic cells. Note that a strip of residual normal endometrium is present (arrow) and shows stronger immunoreactivity than the hyperplastic cells. (F) Both cytoplasmic and nuclear immunoreactivity are noted in the hyperplastical glands. Stromal cells are largely negative. (Original magnification for panel A, C, and E is 10x, for panel B, D, and F is 60x).

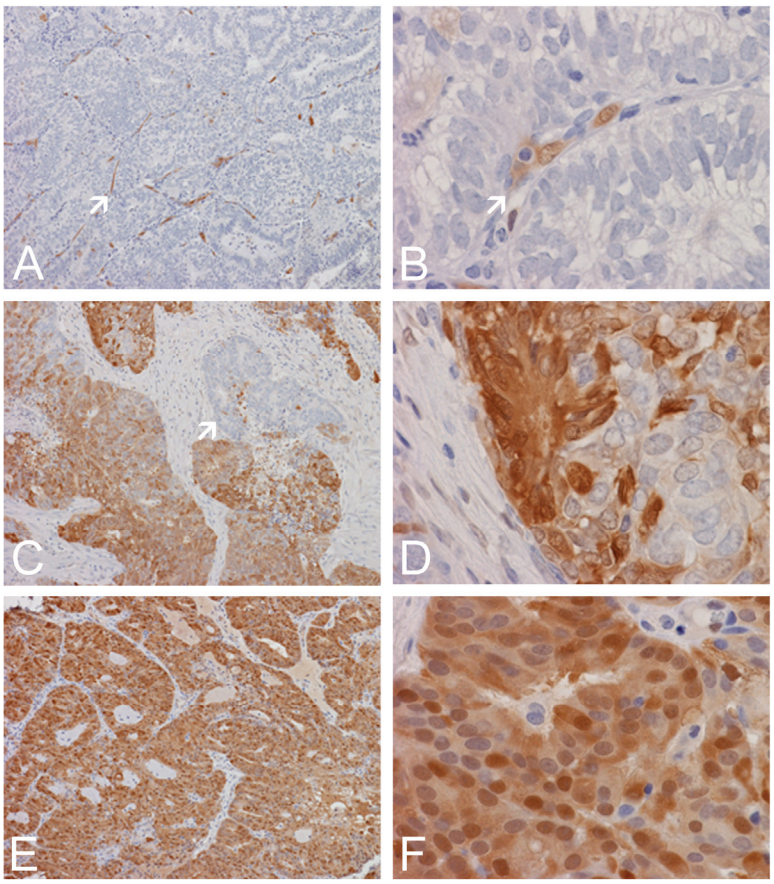

Figure 2: Expression of AKR1C3 in endometrial adenocarcinoma of endometrial type. (A) In this case of adenocarcinoma, there is no immumoreactivity for AKR1C3 in the carcinomatous cells but enodothelial cells of blood vessels are immunoreactive (arrow). (B) Both nuclear and cytoplasmic immunoreactivity are demonostrated in blood vessels (arrow). (C) In this case of adenocarcinoma, there is widespread positive immunoreactivity with small clusters of non-immunoreactive area (arrow). (D) Small clusters of non-immunoreactive cells are present among positive cells. (E) In this case of adenocarcinoma, practically all carcinomatous cells are immunoreactive. (F) Both nuclear and cytoplasmic immunoreactivity are demonstrated. (Original magnification for panel A, C, and E is 10x, for panel B, D, and F is 60x).

Discussion

The expression patterns of AKR1C3 were studied in 13 specimens of endometrium without evidence of hyperplasia, neoplasia or atrophy, 8 specimens of hyperplasia with and without atypia, and 12 cases of primary endometrial adenocarcinoma arising from the endometrium. All of the specimens were obtained from biopsies. A strong, 100% immunoreactivity was demonstrated in the endometrial epithelium without hyperplastic or neoplastic changes. Variable degree of attenuated immuoreactivities was demonstrated in endometrial epithelium with hyperplasia and neoplasia (adenocarcinoma). Consistently, the intensities of immunoreactivity in hyperplastic and neoplastic endometrial epithelium are lower than that of endometrial epithelium without evidence of hyperplastic or neoplastic changes. The endometrial stromal cells are uniformly negative; and there is no difference in immunoreactivities in the stromal cells among the three types of specimens. These results suggest that the levels of AKR1C3 expression are reduced in hyperplastic and neoplastic epithelium.

Our data are different from previous reports [29, 31]. Ito *et al*. showed that AKR1C3 immunoreactivity is detected in 50% and 69% of endometrial hyperplasia and endometrial carcinoma, respectively, as compared to 19% and 25% immunoreactivity in proliferative and secretory phases of endometrium, respectively [29]. In his paper, the authors did not describe the number regarding to the normal endometrium nor did the authors provide any photomicrographs of negative examples. In another study, Šmuc *at al.* demonstrated up-regulated expression of AKR1C3 in endometrial adenocarcinoma [29, 31]. The authors claimed that up regulation of AKR1C3 in 8 out of 16 samples of adenocarcinoma based on levels of mRNA. However, the ratio of epithelial cells to stromal cells is greatly increased in adenocarcinoma and many of these tumors do not contain significant amount

of stromal cells as compared to normal endometrium. As a result, there wound be more mRNA in carcinoma tissue per unit weight even though the level of total RNA per cell remains the same as normal endometrial epithelium. In their study of 16 specimens, there were 6 cases with significant increase, 4 with significantly decreased expression, and 6 with minimal increase or decrease in expression if the cut off point is set at RNA ratio for tumor and normal at 5 (for increased expression) and 0.5 (for decreased expression) respectively. Stastistically, this is not an evidence of increased expression. [31]

Intratumoral steroid hormone metabolism and biosynthesis is important in the etiology and progression of endometrial adenocarcinoma. *In situ* estrogen metabolism, including synthesis and catabolism, has recently been thought to play important roles in the development and progression of various human estrogen-dependent neoplasms including endometrial cancer. These reports have shown that local estrogen biosynthesis can be regulated by aromatase and types 1 and 2 17β-HSD in endometrial carcinoma. Intratumoral production of estrogen occurs as a result of the aromatization of androgens such as testosterone into estrogens; and this reaction is catalyzed by the cytochrome *P*450 aromatase enzyme [32] in the stromal cells or fibroblasts of endometrial carcinoma [33]. The reversible conversions of 17β-estrodiol and estrone can be catalyzed by types 1 and 2 17β-HSD. The 17β-reduction of biologically less active estrone is catalyzed to 17β-estrodiol by type 1 17β-HSD [34], and the oxidation of 17β-estrodiol to estrone is catalyzed by type 2 17β-HSD [35]. It was reported that type 1 17β-HSD immunoreactivity and mRNA were absent in normal and hyperplastic endometrium and in endometrial carcinoma [36, 37], and type 2 17β-HSD expression was detected in normal endometrium (secretory phase) but was decreased in hyperplastic endometrium and endometrial carcinoma [37].

AKR1C3 has also been shown to metabolize progesterone. Based on radiochemical assays, AKR1C3 interconverts progesterone and 20α-hydroxyprogesterone [17]. Protective roles of progesterone in anti-endometrial cancer remain undefined. It has been suggested that progesterone exerts a potent anti-estrogenic effect in epithelial cells of the endometrium by inducing type 2 17β-HSD expression [38]. In contrast, although a strong positive correlation between type 2 17β-HSD and PR expression in the cytoplasm and the nuclei of endometrial carcinoma cells [37], the same report also showed a suppressed expression of type 2 17β-HSD in carcinoma. Since AKR1C3 may regulate the ligand availability for the PR, AKR1C3-mediated progesterone metabolism may affect estrogen action, but require further validation.

Androgens (i.e. androstenedione and 5α-dihydrotesterone), androgen metabolizing enzymes (i.e. 5α-reductases) and the AR have been identified in the endometrium [39]. AKR1C3 can convert androstenedione to testosterone [15]; and this pathway has been suggested for estrogen accumulation in the diseased endometrium [40]. However, with lower AKR1C3 expression in endometrial cancer, this pathway may be impaired in endometrial adenocarcinoma. The role of AKR1C3-mediated androgen conversion in regulating other steroid hormones metabolism in endometrial adenocarnoma requires further study.

The orphan nuclear receptor PPARγ plays important roles in the regulation of lipid homeostasis, adipogenesis, insulin resistance, and the development of various organs. AKR1C3 has 11-ketoprostaglandin reductase activity and forms 9α, 11β-$PGF_{2\alpha}$, and the reaction depletes PGD_2 substrate available for converting to 15-deoxy-$\Delta^{12,14}$-PGJ_2, the naturally occurring PPARγ ligand [41]. Ito *el al*. reported that that PPARγ immunoreactivity is significantly lower in endometrial adenocarcinoma as compared to secretory phase endometrium and endometrial hyperplasia [1]. Although PGJ_2 inhibits cell proliferation in endometrial carcinoma cell lines [42] and $PGF_{2\alpha}$ stimulates endometrial cancer cell line growth and aggressiveness through elevated expression of VEGF [43], roles of altered AKR1C3 expression in regulating PG metabolites in endometrial adenocarcinoma need to be further evaluated.

Based on enzyme kinetics, AKR1C3 catalyzes conversions between estrone and 17β-estrodiol, progesterone and 20α-hydroxyprogesterone, androstenedione and testosterone [17]. In addition, AKR1C3 can reduce 5α-dihydrotestosterone (5α-DHT) through its 3α-HSD activity [14] and accumulate 9α, 11β-$PGF_{2\alpha}$ through its 11-ketoprostaglandin reduc-

tase activities [16]. Based on these observations, AKR1C3 is capable of metabolizing multiple steroid hormones (estrogen, progesterone, androgen, and PG); and reduction or accumulation of these steroid metabolites may have significant impacts on intratumoral hormone balance with altered expression of AKR1C3 in endometrialadenocarcinoma. AKR1C3-mediated steroid hormone metabolisms and their consequences of pathological development remain undefined, and need to be further studied in the development of endometrial cancer.

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Abbreviations: AKR, aldo-keto reductase; AR, androgen receptor; ER, estrogen receptor; HSD, hydroxysteroid dehydrogenase; PG, prostaglandin; PR, progesterone receptor; PPAR, peroxisome proliferator activating receptor.

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