Modulation of estrogenic action in clear cell carcinoma of the ovary (Review)

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Abstract. Two histologic types, clear cell carcinoma (CCC) and endometrioid adenocarcinoma (EAC), are the common histology in ovarian cancer patients who have associated endometriosis. However, both tumor types have distinct clinicopathological characteristics and molecular phenotypes. EAC is predominantly positive for estrogen receptor (ER), but CCC specifically exhibits lower ER expression. This study reviews the current understanding of the role of the ER information in the pathogenesis of CCC, as well as the English language literature for biochemical studies on ER expression and estrogenic action in CCC. The iron-mediated oxidative stress occurs due to repeated hemorrhage in endometriosis, then this compound oxidatively modifies genomic DNA and, subsequently, ER depletion may be observed. There are a number of factors that interfere with ER expression and estrogen activity, which include DNA methylation of the promoter region, histone deacetylation, heme and iron binding, chromatin remodeling and ubiquitin ligase activity. Loss of estrogen function may be a turning point in CCC progression and aggressiveness.

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1. Molecular genetics in endometriosis-associated ovarian cancer

Epithelial ovarian cancer (EOC) remains the number one cause of death from gynecological malignancies. More than 50% of the patients relapse within a few years. Among the EOC, the most common morphological subtype is serous adenocarcinoma (SAC), with less common subtypes including clear cell (CCC), mucinous (MAC) and endometrioid (EAC), generally believed to originate from the ovarian surface epithelium, distal fallopian tube epithelium or peritoneal mesothelial cells. In Japan, however, CCC is not uncommon and is the second most frequent (22%) type of EOC (1). This tumor demonstrates a clinical behavior different from that of the most common histotype SAC. Patients with CCC have early stage with a large pelvic mass, less ascites, chemoresistance and higher incidence of endometriosis and thromboembolism (2). The survival rates of patients with advanced CCC are lower than those of patients with advanced SAC.

Although two histologic types, CCC and EAC, are the common histology in ovarian cancer patients who have associated endometriosis (also known as endometriosis-associated ovarian cancer; EAOC) (3), both tumor types have also distinct clinicopathological characteristics and molecular phenotypes (4,5). The exact mechanisms that turn a process of endometriosis into a cancer precursor are recent topics of intense research. Animal models that resemble the EAOC are of paramount importance in studying the carcinogenesis of these diseases. Several methods for modeling EAC in animals are available. A variety of molecular events, such as PTEN (phosphatase and tensin homolog) silencing and KRAS (v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog) mutations/activation, have been frequently identified in EAC (6). Despite advancements in the molecular analyses of EAOC and the development of animal models, the mechanisms that underlie the pathogenesis of CCC remain largely unknown. Representative CCC animal models have not been available to date. The current knowledge on the mechanisms involved in CCC carcinogenesis shows that ovarian hemorrhage or retrograde menstruation carries highly pro-oxidant factors, such as heme and iron, into the ovarian endometrioma or peritoneal cavity (7). The persistence of a redox active iron in the blood at the site of endometriosis development is important to
understand both induction and promotion of the EAOC. Taken together, DNA damage or loss of heterozygosity (LOH) in an ectopic endometrium caused by iron-induced oxidative stress may be a critical factor in the carcinogenic process (8-11).

CCC carries worse prognoses than EAC tumors. EAC was predominantly positive for estrogen receptor (ER), but CCC specifically exhibits lower ER expression. Aberrant DNA methylation of CpG islands has been recognized in carcinogenesis as a common alteration associated with the loss of expression of a number of key regulatory genes. Many studies have found that the loss of ER expression is a result of the hypermethylation of the ER-α promoter. Although hypermethylation is one of the best known epigenetic events, other epigenetic events, such as histone modification (deacetylation and methylation of histones), are involved in the complex mechanism that regulates promoter transcription, and drive stable, clonally propagated changes in gene expression. The exact interplay of these factors in transcriptional repression activity in CCC is not yet well understood. This study reviews the current understanding of the role of the ER information in the pathogenesis of CCC.

2. Materials and methods

The present article reviews the English language literature for biological, pathogenetic and pathophysiological studies on endometriosis and EAOC. We searched PubMed electronic database for a 20-year period (1990-2010), combining the keywords ‘genome-wide’, ‘microarray’, ‘proteomics’, ‘estrogen receptor’, ‘oxidative stress’, ‘ROS’, ‘carcinogenesis’, ‘iron’, ‘HNF-1α’, ‘mutation’ and ‘chromatin remodeling’ with ‘endometriosis’, ‘ovarian cancer’, ‘clear cell carcinoma’ or ‘endometriosis-associated ovarian cancer’. Several recent studies are discussed in the context of pathogenesis of CCC and DNA mutations. Additionally, references in each article were searched to identify potentially missed studies for a 20-year period.

3. Article selection, data extraction and assessment

Although the main interest is the regulation of ER expression obtained from human ovarian cancer samples, we have included the in vitro studies in the knowledgebase. We also included the animal models performed to support human data. Initially, 58 potentially relevant studies were identified by screening electronic databases. A total of 21 peer-reviewed journal articles were additionally identified from references in each article.

4. Estrogen receptor expression and prognosis in clear cell carcinoma

Estrogen plays a role in ovarian tumorigenesis. In vitro studies indicate that ovarian cancer cell growth is dependent on estrogen stimulation. In a case of breast cancer, ER-negative tumors fail to respond to endocrine therapy and have a poor overall prognosis when compared to ER-positive tumors. The ER-α mRNA level was a significantly positive prognostic factor for patient survival. The prognostic significance of ER expression by ovarian cancers has received little attention to date. One study reported a correlation between levels of ER expression and cancer disease stage, with levels declining with increased severity of disease, suggesting that loss of ER expression in ovarian cancer is a feature of malignant transformation and aggressiveness (12). Despite the poor prognosis of patients with ER-negative disease, there remains considerable heterogeneity in individual outcomes. Indeed, other studies demonstrated that since ER has emerged as a mitogenic factor, ER status is a prognostic factor for ovarian cancer with better survival for ER-negative tumors (12). Furthermore, a higher ER expression at the mRNA and protein levels was found to be associated with a longer progression-free survival and overall survival (13). The remaining investigators reported, however, that neither ER nor progesterone receptor (PR) independently correlated with survival in the overall study population. Therefore, there is a controversy as to whether ER expression is a prognostic factor for outcomes in ovarian cancer.

Among EAOC, EAC were predominantly positive for ER and PR (14), but CCC specifically exhibited lower ER and PR expression (14). The investigators put forward a model postulating that additional events, particularly deletion of ER expression, are required for CCC lesion progression. CCC pathogenesis may be a model to study the disease progression from estrogen-dependent to estrogen-independent, allowing design of new strategies targeting the hormone response, thereby modifying disease outcome. Therefore, loss of estrogen function may be a turning point in CCC development. There are basically the following hypotheses regarding the carcinogenesis or pathogenesis of CCC: initially, the heme and iron-mediated oxidative stress and persistent inflammation processes occur due to repeated hemorrhage in endometriosis. These compounds oxidatively modify DNA, proteins and lipids, and subsequently hypermethylation or ER depletion may be observed (Fig. 1). Suzuki et al reported that ER is inactivated mainly through aberrant DNA methylation (15).

A dualistic model, which has been established on morphological and genomic basis, differentiates EAOC into two broad categories: estrogen-dependent ovarian cancers with an EAC morphology, and estrogen-independent carcinoma with the CCC morphology (4). The genetic pathways utilized by ER-negative tumors to proliferate in the absence of a mitogenic estrogen signal are poorly understood. Elucidation of these pathways is required for the development of improved therapies for ER-negative CCC patients.

5. Factors contributing to the expression of estrogen receptor

Estrogen is supposed to actively participate in the early stages of endometrial tumorigenesis. ER also plays a role in the onset and development of tumors arising from or outside the reproductive system. Genomic aberrations of ER-dependent downstream targets provide cellular growth advantages when the cells are exposed to estrogen. There are a number of factors that interfere with ER expression and estrogen activity. Many factors are implicated as an important regulator of distinct aspects of ER action and expression, including gene expression, DNA methylation of the promoter region, histone deacetylation and heme and iron binding (16) (Fig. 1).

Methylation of the ER gene. DNA damage by iron-mediated oxidative stress could be one factor leading to the development

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of EAOC (9,10). Levels of the oxidatively modified guanosine base 8-oxoguanine (8-oxoG) were increased in the endometriotic cells (17) and CCC cells (8). Oxidative stress activates signaling cascades that lead to induction of stress-responsive genes (genetic alteration). In addition to the genetic alterations, evidence supports a role of oxidative stress in the epigenetic process. DNA methylation is among the most studied epigenetic mechanisms (18). Beside the standard picture of DNA methylation, factors contributing to the outcome of oxidative damage to nucleic acids may be chromosomal aberration, microsatellite instability and telomere shortening (19). Several investigators focus on DNA methyltransferases (DNMT) and histone deacetylases (HDAC) as epigenetic targets for cancer treatment. Such lesions of genome DNA damaged by oxidative stress interfere with the ability of DNA to function as a substrate for DNMTs, resulting in hypomethylation (19). Loss of methylation in normally methylated sequences (hypomethylation) may lead to activation of HNF-1β and genomic instability, which is evident in CCC.

In addition, a growing body of evidence has accumulated that DNA methylation and various histone modifications involved in chromatin remodeling have been linked to the lack of ER expression (15,20). This change may lead to inappropriate methylation in the promoter region of ER gene. Methylation of the ER gene has been linked to breast, colon, prostate and hematopoietic malignancies (20). The ER promoter is also methylated in ovarian cancer cell lines and tissue samples (15). These results indicate that aberrant hypermethylation is responsible for a significant proportion of EAOC in which ER expression is lost. This process may play an important role in the pathogenesis of CCC.

Histone deacetylase (HDAC). There are several genes that regulate ER transcription. Histone acetyltransferases (HAT) and HDACs cause post-transcriptional modification of histone proteins that participate in ER signaling. HDAC exerts specific roles in breast cancer progression and estrogen dependence, because this family suppresses ER transcriptional activity (21). HDAC inhibitor induces stabilization and expression of ER gene and restores sensitivity of the ER-negative cancer to chemotherapy. Therefore, several investigators focus on HDAC as epigenetic targets for cancer treatment. HDAC may be a potential target for therapeutic approach or intervention in the treatment of a subset of ER-negative cancers, including CCC.

Furthermore, of interest is the observation that metastasis-associated protein 1 (MTA1) acts as a potent corepressor of ER in breast cancer cells (22). MTA1 is the component of a nucleosome remodeling deacetylase complex. This gene represses ER transcription by recruiting HDAC to the ER response element-containing target genes, which is accomplished by chromatin remodeling. MTA1 overexpression also results in down-regulation of E-cadherin and overexpression of Snail and Slug, leading to the epithelial mesenchymal transition and enhancing invasion and metastasis (22). Thus, MTA1 may be a predictor of aggressive phenotypes. It remains unclear, however, whether CCC retains a similar cellular activity on the HDAC-regulated ER expression to that found within breast cancer.

Figure 1. Hypotheses regarding the carcinogenesis of CCC: factors contributing to the expression of ER. Estrogen plays a role in ovarian tumorigenesis. Among EAOC, EAC was predominantly positive for ER and PR, but CCC specifically exhibited lower ER and PR expression. A dualistic model, which has been established on morphological and genomic basis, differentiates EAOC into two broad categories: estrogen-dependent ovarian cancers with an EAC morphology, and estrogen-independent carcinoma with a CCC morphology. There are basically the following hypotheses regarding the carcinogenesis or pathogenesis of CCC: initially, the heme and iron-mediated oxidative stress and persistent inflammation processes occur due to repeated hemorrhage in endometriosis. These compounds oxidatively modify DNA, proteins and lipids and, subsequently, ER depletion or hypermethylation may be observed. Loss of estrogen function may be a turning point in CCC development. Many factors are implicated as significant regulators of distinct aspects of ER action and expression, including gene expression, DNA methylation of the promoter region, histone deacetylation and heme and iron binding, PPARγ and ubiquitin protein ligase. The switch from a normal-stress-response phenotype to a stress-resistant phenotype may involve the mutation of the specific genes in CCC. Additional candidate gene (e.g., ARID1A) abnormalities or molecular alterations may cause the progression towards the CCC.
Iron. In an excess of a redox active iron, this metal is easily incorporated into the zinc fingers of ER molecule by means of a zinc/iron exchange, because zinc appears to be kinetically labile and is exchangeable. Iron fingers of ER molecule will augment the rate of hydroxyl radical generation (e.g., hydroxyl radicals via Fenton reaction) and enhance oxidative stress (23). Iron-induced free radical formation and subsequent oxidative stress lead to extensive damage to the proximate DNA, e.g., the estrogen responsive element of ER, which results in suppression of ER expression. Iron-induced hydroxyl radical generation also causes adverse consequences, such as carcinogenesis (23).

Heme. Similar to iron, heme appears to be rich in endometriotic cyst fluid and peritoneal fluid. Heme is a physiological ligand of NR1D2 (nuclear receptor subfamily 1, group D, member 2), belonging to the steroid receptor superfamily of transcription factors (24). Heme binding to NR1D2 causes the recruitment of the co-repressor NCOR1 (nuclear receptor co-repressor 1) and SMRT (silencing mediator for retinoid and thyroid-hormone receptors). SMRT contribute to the corepressor recruitment and modulation of ER transcriptional activity. These co-repressors are modulated by the ER's recognition of cognate DNA binding sites. This complex also leads to repression of target genes, including ER, by promoting chromatin condensation and recruitment of HDAC complexes (24). The differential expression pattern of ER and its co-activator/co-repressor proteins may fine-tune the modulation of estrogenic action. The up-regulation of NCOR suppresses estrogen-induced growth in the ER-positive cancer cells. High NCOR mRNA levels were associated with a better prognosis, demonstrating that the NCOR level may affect prognosis among women with breast cancer. In addition, recent data suggest that some co-repressors, other than NCOR/SMRT, may be involved in ER signaling. The precise role in heme-dependent regulation of ER expression remains unclear in CCC.

Peroxisome proliferator-activated receptor (PPAR) γ. Nuclear receptors are transcription factors that can be activated by specific ligands (25). On activation by a ligand, nuclear receptors recruit the retinoid X receptor (RXR) for heterodimer formation (25). Such nuclear receptors include the peroxisome proliferator-activated receptor (PPAR) γ. The PPARγ/RXR heterodimer binds SWI/SNF (mating type switching/sucrose non-fermenting) chromatin complex (26). The SWI/SNF complex facilitates gene transcription by remodeling chromatin using the energy of ATP hydrolysis. This complex always contains either BRG-1 (brahma-related gene 1; hSNF2β) or BRM (brahma; hSNF2) as the catalytic subunit, together with associated factors (BAFs, BRG1-associated factors) (26). BRG-1 and BRM enhance the transcription of genes regulated by several transcription factors, including ER (26). The SWI/SNF complex reportedly regulates many important genes involved in energy homeostasis (diabetes and atherosclerosis) (25). Major rearrangement of the nucleosomal chromatin facilitates recruitment of BAFs, which have ten isoforms, including BAF250a (26). BAFs regulate transcription of certain genes by altering the chromatin structure around

<table>
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<td>Cysteine-rich PAK1 inhibitor</td>
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<td>Estrogen-related receptor β</td>
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<tr>
<td>Glutathione S-transferase M3 (brain)</td>
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<tr>
<td>Estrogen-related receptor β</td>
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</tr>
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<tr>
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<tr>
<td>Hydroxysteroid (17-β) dehydrogenase 2</td>
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<tr>
<td>UDP glucuronosyltransferase 2 family, polypeptide B28</td>
<td>-1.45</td>
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</table>

Microarray database of HNF-1β knockdown vs. parental KOC7c ovarian cancer cells revealed altered expression of 19 genes known to be involved in the estrogenic action and ER expression.
those genes (26). For example, BAF250a has a DNA-binding domain that specifically binds an AT-rich DNA sequence and stimulates glucocorticoid receptor-dependent transcriptional activation. BAF250a is also known as ARID1A (AT rich interactive domain 1A) (27) (Fig. 1). BAF250 forms an E3 ubiquitin ligase that targets histone H2B (27). H2B ubiquitination plays critical roles in regulating many processes within the nucleus, including transcription initiation and elongation, silencing and DNA repair. The loss of function of BAF250 due to somatic or genetic mutation may fail to ubiquitinate the H2B molecule, which results in aberrant chromatin remodeling.

More recent data for the first time showed that the specific genes that were somatically mutated in CCC are ARID1A, PIK3CA (phosphoinositide-3-kinase, catalytic, α polypeptide), PPP2R1A (protein phosphatase 2, regulatory subunit A, α) and KRAS (v-Ki-ras2 Kirsten rat sarcoma viral oncogene homolog) (28,29). Nearly half the CCC carried truncating mutations in ARID1A (BAF250a) (28,29). These investigators propose that aberrant chromatin remodeling contributes to the pathogenesis of CCC (28,29). The mechanism of action of ARID1A and the SWI/SNF family complex is interesting to consider. ARID1A may contribute to the development of CCC, possibly through a lack of tumor suppressor gene and suppression of estrogenic action.

High expression level of PPARγ correlates with long-term survival in patients with breast cancer. PPARγ expression is also positively correlated with ER status. These observations correspond to estimations that up-regulation of HNF-1β expression in CCC down-regulates ER expression via suppression of PPARγ. The PPARγ agonist troglitazone (TRG; anti-diabetics) may suppress P13K signaling through HDAC inhibition, leading to increased histone modifications. Estrogen-related receptor α and γ (ERRα and ERRγ) are alternative targets of TRG important for mediating its growth suppressive effect. Therefore, it is certainly possible that ARID1A and its downstream targets may be a therapeutic target for CCC.

Ubiquitin protein ligase E3A (UBE3A, E6-AP). Other potential mechanisms underlying ER generation involve E6-AP (UBE3A, ubiquitin protein ligase E3A) (30). This gene encodes an E3 ubiquitin-protein ligase, part of the ubiquitinprotein degradation system. The E6-AP expression is inversely associated with that of ER, suggesting that E6-AP suppresses the ER expression. This gene has been shown to be elevated in ER-negative breast cancer and drives the protesomeal degrada
tion of ER. In addition, E6-AP regulates cell proliferation by promoting proteasome-mediated degradation of cyclin-dependent kinase inhibitor p27.

6. Conclusion

Epidemiologically and clinicopathologically, EAC and CCC are believed to develop from endometriosis (EAOC). Under certain conditions in endometriosis, the biological behavior change of the local microenvironment may promote the development of EAOC. Recent studies have demonstrated that a redox active iron overload and the accompanying chronic inflammatory processes will supply critical initiators inducing cell growth, survival, anti-apoptosis and finally the oxidative stress-response, leading to carcinogenesis. Among EAOC, CCC differs from EAC with respect to their clinical characteristics and carcinogenesis (10). A growing body of evidence has accumulated that, compared to EAC, CCC is relatively resistant to conventional taxane plus platinum-based chemotherapy (31). Therefore, EAC shows favorable prognosis, whereas CCC contributes to poor prognosis. There is a review focusing on the specific gene expression that may predict the response to chemotherapy and/or prognosis (7).

The genome-wide expression analyses identified differences in the expression of several genes and proteins between CCC and EAC (4,5,7-11,32). The literature data on the expression of ER in CCC differ from that in EAC (14). Compared to EAC, CCC exhibits very low ER expression. CCC characterized by ER negativity may be an important factor for an aggressive tumor with poor prognosis. Several studies have defined sets of genes with differential expression levels between ER-positive and ER-negative tumor types. For example, ER-negative breast cancer exhibits a greater proliferation signal, failure of chemotherapy and an aggressive tumor with poor prognosis. We put forward a model postulating that deletion of ER expression is required for CCC lesion progression and aggressiveness.

The present review demonstrated that many factors are implicated as critical regulators of distinct aspects of estrogenic action and ER expression, including gene overexpression and silencing, DNA methylation of the promoter region, histone deacetylation, heme and iron binding, PPARγ action and ubiquitin ligase activity. Consequently, loss of ER expression may be associated with a specific additional risk for developing CCC, but not EAC. Our preliminary microarray data on the HNF-1β siRNA experiments support the notion that the estrogenic action and ER expression are down-regulated in response to HNF-1β (Shigetomi et al, 2011, unpublished data). The up-regulation of HNF-1β expression led to subsequent modulation of its downstream targets that directly or indirectly suppress the estrogenic action. We have defined the gene set in CCC, with a view to identify the HNF-1β-dependent estrogenic action. Some of the genes predominantly identified in CCC were related to ER modulation, which may be involved in downstream targets of HNF-1β gene. This result is particularly interesting because CCC cells that show overexpression of HNF-1β gene may down-regulate the estrogenic action and ER expression.

More recent data showed that the specific genes that were somatically mutated in CCC are ARID1A, PIK3CA, PPP2R1A and KRAS (28,29). ARID1A mutation results in aberrant chromatin remodeling and may contribute to the development of CCC, possibly through a lack of tumor suppressor gene (28,29) and suppression of estrogenic action. Additionally, PPP2R1A is another gene that exhibits acquired somatic mutations in the genome in CCC (29). PPP2R1A functions, when mutated, as an oncogene. PPP2R1A is a regulatory subunit A-α of PP2A (serine/threonine protein phosphatase type 2A). PP2A plays an essential role in cell cycle regulation and induction of G2 arrest by a mechanism of phosphorylation/dephosphorylation with a variety of protein kinases. Our preliminary data showed that PPP2R1A (29) and PP2A can be deregulated in CCC either by somatic mutation and transcriptional repression by HNF-1β, respectively (Shigetomi et al, 2010, unpublished data). Furthermore, the PP2A complex is also associated with ER and leads to its dephosphorylation and inhibiting transcription.
HNF-1β is differentially expressed among the EAOCC. The nuclear immunostaining of HNF-1β protein is observed in the majority of CCC, but not in EAC, demonstrating that this aberration is histotype-specific. Recent biochemical studies based on genome-wide microarray analysis technology have noted specific expression of HNF-1β in CCC (33). Kato et al reported that hypomethylation of the HNF-1β CpG island participates in the HNF-1β up-regulation in CCC (34). Among the genes highly up-regulated in CCC, more than 80% genes were associated with the redox-related gene (32). Several important CCC-related genes overlap with those known to be regulated by HNF-1β (32).

Several investigators have described the HNF-1β-dependent pathophysiology of CCC and discussed its role in oxidative stress-induced carcinogenesis (7-11). A majority of CCC likely develops as the result of a stepwise accumulation of alterations in cellular regulatory pathways, such as DNA methylation, genomic DNA mutation, LOH, oncogene activation, tumor suppressor gene inactivation and aberrant chromatin remodeling. The gene expression profiling and signaling pathways of HNF-1β, so called ‘HNF signature’, has been assessed in CCC: the catalog of CCC-specificity may be a manifestation of several essential alterations in cell physiology, including oxidative stress, detoxification and metabolism (7,8). The ‘HNF signature’ shows evidence of biological functions, including glycogen storage (energy accumulation), anti-apoptosis (survival) and detoxification (stress-resistance).

Up-regulation of HNF-1β expression was evident in the CCC and contiguous atypical endometriosis and even in distant endometriotic lesions (35). A candidate early precursor to CCC, the ‘HNF signature’, is commonly found even in endometriosis in the absence of any morphological abnormality (35-38). The ‘HNF signature’ may be an early event of the clear cell directionality (4,7-10,32). Therefore, up-regulation of HNF-1β expression is not sufficient for the development of CCC. Birrer proposed that the switch from a normal-stress-response phenotype to a stress-resistant phenotype may involve the mutation of the specific genes in CCC (39). Additional candidate gene abnormalities or molecular alterations may cause the progression towards the CCC. ARID1A mutations are evident in the CCC and contiguous atypical endometriosis, but not in distant endometriotic lesions (28). Therefore, ARID1A is considered to be an early event in the transformation of endometriosis into ovarian cancer (28).

In conclusion, this review examines the suppression of estrogenic action and ER expression in CCC. The basic findings of this comprehensive review suggest that the interactions between the iron-mediated oxidative stress and down-regulation of ER expression may have clear implications for developing CCC via the up-regulation of HNF-1β expression. These genes and proteins significantly elevated and repressed in CCC subjects may provide a foundation for further validation in larger patient cohorts. Future studies will address the mechanism of synergistic effects of the specific gene mutations and up-regulation of HNF-1β gene and its downstream target genes on the estrogenic action. Thus, targeting HNF-1β-mediated signaling may serve as a novel therapeutic strategy for the treatment of CCC. Certain inhibitors are currently being studied as new drugs able to restore ER-α protein expression in ER-α-negative cancer cells and to promote apoptosis and differentiation. Demethylating drugs and HDAC inhibitors are candidates for becoming potent new drugs in cancer therapy.

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