Androgen Receptor Increases CD133 Expression and Progenitor-Like Population That Associate With Cisplatin Resistance in Endometrial Cancer Cell Line

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Lumin Chen, MD^{1,2}, Wei-Chun Chang, MD², Yao-Ching Hung, MD, PhD^{1,2}, Ying-Yi Chang, MD², Bo-Yin Bao, PhD^{2,3}, Hsin-Ching Huang, MS², Wei-Min Chung, MS^{1,2}, Chih-Rong Shyr, PhD^{1,2}, and Wen-Lung Ma, PhD^{1,2}

Abstract

Endometrial cancer (EMC) is a sex steroid hormone-related female malignancy. Androgen and androgen receptor (androgen/AR) signals have been implicated in EMC progression. Cancer stem/progenitor cells (CSPCs) are suspected to link to chemoresistance in patients with EMC. In this study, we examined the androgen/AR roles in cisplatin resistance and CSPC population. We found AR expression increased naive EMC side population, CSPC population, cell migration, and epithelial-mesenchymal transition. Meanwhile, it decreased cisplatin cytotoxic effect on EMC cells. Collaterally, endogenous AR expressions in EMC cells were upregulated in the cisplatin-resisting state. Moreover, AR expression could further enhance CD133 expression, CSPC-related markers, and drug-resistance gene messenger RNA expression in EMC cells. Finally, the AR-associated gene expression might go through indirect regulation. This is the first report revealing AR function on EMC cells' CSPC and cisplatin resistance.

Keywords

AR, endometrial cancer, cancer stem/progenitor cells, cisplatin resistance, side population cell

Introduction

Endometrial cancer (EMC) is one of the most common female malignancies worldwide.¹ In the United States, estimations show that there are 42 100 annual new cases and 7400 deaths caused due to this disease. During the past 30 years, there is no significant improvement in the survival rate of patients with advanced EMC.² Except for curative surgery of tumor resection, chemotherapy reagents are considered as alternative defending treatments.³ Cisplatin is one of the most commonly used agents in the postcurative surgery in patients with EMC. Yet, only 25% to 35% patients are cisplatin responsive in this category,^{4,5} implicating high heterogeneity and significant population of cisplatin-resistant cells in the tumors.

In order to accommodate this heterogeneity, a relatively quiescent cancer stem/progenitor cells (CSPCs) were allowed to escape the cytotoxic effects of chemotherapeutic agents that target rapidly cycling cells.⁶⁻⁸ It has been identified that surface marker protein CD133+ cells exhibited CSPC characteristic,⁶ which is recognized as a CSPC marker.

Androgen and androgen receptor (androgen/AR)⁹ signals have been implicated in the carcinogenesis and progression of EMC. Earlier study using AR knockout mouse¹⁰ has revealed diminished endometrial growth.¹¹ One study claimed that androgen/AR signal could promote EMC disease phenotype in vivo.¹²

Furthermore, the expression¹³ and shorter CAG tract (implicating higher AR transactivation function) of the AR gene¹⁴ have been associated with EMC cancer malignancy. Besides, female-to-male transsexual patients show an increased risk of EMC, which is associated with higher serum testosterone levels and AR expression in endometrium.¹⁵ Since AR has been implicated in the EMC carcinogenesis and cancer progression, whether it participates in the therapy resistance is still not yet examined. In order to approach the androgen/AR roles in CSPC, Ishikawa EMC cells (high CD133+ expressing cells⁶) were introduced in this study.

¹ Sex Hormone Research Center, Graduate Institution of Clinical Medical Science, School of Medicine, China Medical University, Taichung, Taiwan

² Department of OB/GYN, China Medical University Hospital, Taichung, Taiwan

³ Department of Pharmacology, China Medical University, Taichung, Taiwan

Corresponding Author:

Wen-Lung Ma, Sex Hormone Research Center, Graduate Institution of Clinical Medical Science, School of Medicine, China Medical University Hospital, 505 Rm, 6 Xueshi Rd, North District, Taichung City, 404, Taiwan.

Email: maverick@mail.cmu.edu.tw

Materials and Methods

Cell Line Maintenance, Plasmids, and Reagents

The Ishikawa EMC cells¹⁶ were kindly provided by Professor C. Y. Chou from National Cheng Kung University Hospital (Tainan, Taiwan). The cells were maintained in the Dulbecco-modified Eagle medium with 10% fetal calf serum (Invitrogen, Carlsbad, California), 1% L-glutamin, and 1% penicillin/streptomycin as described.¹⁷ The antibody used were AR (N-20; Santa Cruz, Dallas, Texas), E-cadherin (Cell Signaling, Danvers, Massachusetts), vimentin (Santa Cruz), actin (Santa Cruz), CD133-phycoerythrin (PE)-conjugated antibody (MACS, Gladbach, Germany), and immunoglobulin G (IgG)-PE-conjugated isotype (MACS). The chemicals used were cisplatin (Sigma-Aldrich, St. Louis, Missouri), Hoechst 33342 (Sigma-Aldrich), verapamil (Sigma-Aldrich), bovine serum albumin (Sigma-Aldrich), and propidium iodine (Sigma-Aldrich).

Cisplatin Resistance and AR Stable Transfection on Ishikawa Cells

The 1×10^7 cells were treated with sub-half maximal inhibitory concentration (sub-IC₅₀) lethal dose (20 μ mol/L) of cisplatin and incubated for 7 days. After 7-day culture, the cells were recovered for 24 hours and then subjected to assays. For the AR stable clone cells, pBabe-hAR plasmid¹⁷ (wild-type human AR full-length complementary DNA [cDNA] containing plasmid) was used to transfect the Ishikawa cells following previous procedure.¹⁸ Puromycin (2 μ g/mL) was used to select stable transfectant (Ishi-AR), and the pBabe vector transfected cells were used as parental clone (Ishi-par).

Side Population and CD133 Staining to Identify CSPC Cells

Cells were detached by trypsin (1%; Invitrogen), washed, and then 10^6 cells were subjected to 2 flow tubes. For side population (S/P) assay, Hoechst 33342 (5 μ g/mL) and/or verapamil (50 μ mol/L) were added and incubated at 37°C for 90 minutes.^{6,19} After incubation, cells were washed and then subjected to flow cytometry assay using flowcytometer (SLR11, BD; Invitrogen). For CD133 staining,⁶ the cells were harvested to stain with CD133-PE- or IgG-PE-conjugated isotype antibodies then subjected to flow cytometry assay. The data were analyzed using FlowJo 7.6 software (Tree Star, Inc, Ashland, Oregon).

Cell Growth Assays Using WST-1 and Cell Counting and Wound-Healing Migration Assay

The 2×10^3 cells were seeded in 96-well plates and cultured for 0, 2, 4, and 6 days. At the time of assay, 10 μ L/well WST-1 solution (Roche) was added into the 96-well plates, then incubated for 1 hour.²⁰ The optic absorbencies were directly read by PARADIGM detection platform (Beckman Coulter, Brea, California). We also used the automate cell counter (T10; BioRad, Hercules, California) to count the total cell number. The 10^4 cells were plated onto 60-mm dish and incubated for 0, 2, 4, and 6 days. After

incubation, cells were detached and the trypan-blue dye was added to the cell solutions to exclude the dead cells. Only the cells alive were counted. For the wound-healing cell migration assay, 5×10^5 cisplatin-resistant cells were seeded onto the 6-well plates. Allowed cells adhered onto wells for 18 hours and using 200- μ L pipette tip the cells were scratch to create linear wound areas. Then, immediately photo images of the cells were taken at 0 hour. We observed and took photographs of wound closure 48 hours after the wounds were created. The wound areas (μ m²) of 0 hour deducted within 48 hours in the same well is considered as migration activities. The photograph images were analyzed by NIS Elements BR3.1v software (Nikon, Tokyo, Japan).

Gene Expression Assays

Total cell RNA was obtained from the cultured cells using TriZol (Invitrogen) and phenol-chloroform pH 6.7/8.0 (AMRESCO, Solon, Ohio) method. Total RNA of 2 μ g was subjected to reverse transcription using PrimeScript and TaKaRa kit (TaKaRa, Japan). The messenger RNA (mRNA) expression levels of CSPC markers (SCF,²¹ Bmi1,²² Nanog,²³ Oct4,²⁴ Notch,²⁴ CD24,²⁵ CD133,^{6,26} CD44,²⁶ ABCG2,²⁷ CD117,²¹ and Nestin²⁸) and cisplatin-resistance genes (*MDR1*,²⁹ *ABCB5*,³⁰ and *ABCG2*³¹) were determined using quantitative reverse transcription-polymerase chain reaction (Q-RT-PCR) as described previously.³² The cDNAs were subjected to Q-PCR assay using C1000 Cyclex CFX96 Real-time System (BioRad) with iQ SYBR Green Supermix (BioRad). The primers used for Q-PCR were summarized in Supplemental Table 1. The protein expression was analyzed using immunoblotting assay.^{17,32} The reporter gene assays were performed as described previously.¹⁷ The promoter constructs were androgen response element (ARE)(4)-luciferase, and thymidine kinase-driven renilla luciferase (pRL-TK) was served as transfection efficiency control.

Androgen Receptor Binding in the Prediction of Transcription Clusters

The genes of interest were subjected to PReMod³³ (<http://genomequebec.mcgill.ca/PReMod/search/module>) for predicting potential AR-binding sites (module number: M00447). The 10 kbp of 5'-promoter and 5 kbp of 3'-downstream of interested genome were included for prediction.

Statistical Analysis

The results were analyzed using student *T* test for all the experiments, standard deviation served as the experimental variations, and the *P* values of less than .05 were considered to be statistically significant.

Results

Androgen/AR Signal Enhances Basal CSPC Characteristics Without Affecting Cell Growth

In order to examine the AR effects on CSPC characteristics, we first stably transfected AR cDNA in the Ishikawa EMC cells.

As shown in Figure 1A, the Ishi-par cells expressed little AR protein, while stably transfected Ishi-AR cells expressed significantly higher amount of AR (Figure 1A). We also examined the AR transactivation function using dual-luciferase assay (Promega, Madison, Wisconsin) and found that stably transfectant Ishi-AR was around 3- to 4-fold higher than that in Ishi-par cells (Figure 1B). Then the Ishi-par and Ishi-AR cells were parallel compared to the side population using Hoechst inclusion assay.¹⁹ As shown in Figure 1, the side population cells are defined in Figure 1C, and quantitative amount of side population are shown in Figure 1D. We found that Ishi-AR cells contain higher side population while compared to Ishi-par cells. Furthermore, we examined CD133-expressing populations using flow cytometry⁶ to confirm AR effects on CSPC characteristics. As shown in Figure 2, CD133 was expressed in both Ishi-par and Ishi-AR cells. While examining AR effects on CD133 expression, we found the intensity of CD133 in Ishi-AR is around 200-unit stronger than Ishi-par cells (Figure 2A). Paralleled with the intensity of CD133 expression, the populations of CD133 cells in Ishi-AR were 1.2- to 1.4-fold compared to Ishi-par cells (Figure 2B). Since AR could enhance CSPC characteristics, we examined whether AR could influence the overall cell growth. We examined Ishi-par and Ishi-AR cell growth using WST-1 (Figure 3A) and cell counting (Figure 3B) assays and found a decreasing trend of cell growth, yet, no significant change between these two cells. Moreover, we examined cell migration (Figure 3C) and epithelial-mesenchymal transition (EMT) marker expressions (Figure 3D). We found Ishi-AR exerted more migratory capacity compared to Ishi-par cells. And the epithelial marker E-cadherin was downregulated, while mesenchymal marker vimentin was upregulated upon AR expression. Together, this result indicates that AR influences on CSPC characteristics, cell migration ability, as well as EMT transformation but not on cell growth.

Androgen/AR Signal Enhances Cisplatin Resistance and Related CSPC Characteristics

Cancer stem/progenitor characteristics are associated with chemotherapy resistance. Therefore, we proceed to examine the AR effects on cisplatin sensitivity and resistance. We first established cisplatin-resistance Ishi-par and Ishi-AR cells by long-term (14 days) culture using sub-IC₅₀ dose of cisplatin (10 μ mol/L). The AR mRNA expressions in naive and resistance cells were performed using real-time Q-PCR assay. We found that AR mRNA expressions were increased in both Ishi-par and Ishi-AR cells after transforming to cisplatin resistant. In the naive cells, the AR mRNA expression in Ishi-AR was about 10-fold higher than that in Ishi-par cells (Figure 4A; lane 1 vs lane 2). However, the AR mRNA expression of cisplatin-resistant Ishi-AR is around 65-fold higher than that in Ishi-par cells (Figure 4A; lane 3 vs lane 4). Furthermore, we examined the intensity of CD133 expression using flow cytometry and found cisplatin-resistant Ishi-AR CD133 expression is about 400-unit intensity stronger than that in

Figure 1. The AR expressing Ishikawa cells and AR increases the S/P cells. A, Stable transfectant of AR complementary DNA (cDNA) in Ishikawa cells was confirmed by Western blot assay. par: Ishi-par (vector transfectant of Ishikawa cells); AR: Ishi-AR (AR expressing transfectant of Ishikawa cells). B, AR transactivation function measured by androgen response element (ARE)-driven luciferase reporter activity. The Ishi-par and Ishi-AR cells were treated with 10 nmol/L 5 α -dihydrotestosterone (DHT) for 18 hours after cotransfecting with ARE-luciferase/pRL-TK plasmids. The readings of Ishi-AR were compared to Ishi-par cells and presented as fold changes. C, Hoechst incorporation assay by Flow cytometry. The verapamil cotreatment were subjected and compared with the readings of Hoechst treatments only to illustrate S/P. Representative images were presented, and higher S/P was noticed in Ishi-AR than in Ishi-par. D, Quantitative results of (C). All the experiments were calculated from 3 independent experiments. AR indicates androgen receptor; S/P, side population.

Ishi-par cells (Figure 4B). In order to examine the AR effect on cisplatin resistance, we compared IC₅₀ and CD133+ populations in the cisplatin-resistant Ishikawa cells. We found IC₅₀ in cisplatin-resistant cells were increased compared to naive cells (22.9-28.8 μ mol/L), while AR expression increased further (32.5-44.5 μ mol/L; Figure 4C). The CD133+ populations comparing naive and cisplatin-resistant cells were increased in

Figure 2. Androgen/AR enhances basal levels of CD133 expression and population. A, Cell surface protein CD133 expression was measured in Ishi-par and Ishi-AR cells using flow cytometry assay. The par-CD133 and AR-CD133 represented CD133-PE-conjugated antibody staining of cells. The dashed lines (green dash line: par-CD133 and orange dash line: AR-CD133) indicated the mean CD133 fluorescence intensities. B, CD133+ populations in Ishi-par and Ishi-AR cells. The overlapping of isotype IgG-PE histogram graphs in (A) was excluded and the residual areas were counted as CD133+ populations. The data were the mean values from 3 independent experiments. AR indicates androgen receptor; IgG-PE, immunoglobulin G-phycoerythrin.

Ishi-par (17.3% vs 25.3%), while further increased in Ishi-AR (23.2% vs 36.7%; Figure 4C).

In order to further examine AR effects on CSPC, we measured cancer stem/progenitor makers and related gene expression by Q-PCR. As shown in Figure 5, stem/progenitor-related transcription factors were measured and found the SCF, Bmi-1, Nanog, Oct-4, and Notch are higher in Ishi-AR than the differential levels in Ishi-par (Figure 5A). Meanwhile, stem/progenitor surface markers CD24, CD133, CD44, and ABCG2 were also higher in Ishi-AR than in Ishi-par. Using cisplatin-resistant (14 days of treatment) Ishikawa cells comparing AR expression and naive cells, we found MDR1, ABCG2, and ABCB5 were upregulated in Ishi-AR compared to Ishi-par cells (Figure 5C). Furthermore, we analyzed potential AR regulatory modules on those AR-enhanced genes using PReMod database website and found there is no potential AR binding on those gene genomes. This result suggests that AR-related CSPC might go through nonclassical transactivation activity, or a secondary effect on gene expression.

To sum up, androgen/AR signal could promote EMC basal CSPC levels. While cancer cells up-front cisplatin treatments, AR might further enhance cells' resistance to therapy via increasing CSPC.

Discussion

CD133 and Side Population Cells as EMC Progenitor Markers Associated With Cisplatin Chemoresistance

Recent phase II cisplatin clinical trial conducted in Japan showed limited survival benefit on low- to intermediate-risk group defined as patients with stage IC EMC. However, higher

grades (stage II or IIIA) exert significant survival benefit, implicating cisplatin reagent effect on patients with EMC.³⁴ Recent review article summarized CD133 and side population detections are potential EMC stem-progenitor marker.³⁵ In this article, Kyo provided perspective to the value of CD133 and S/P cells as potential EMC carcinogenesis mechanism. Nakamura et al showed CD133 population is associated with cisplatin or paclitaxel resistance.⁶ CD133+ cells showed apparent resistance to cisplatin- or paclitaxel-induced cytotoxicity and higher S-phase fraction than CD133(−) cells. In the current study, AR can promote CD133+ and S/P cells, which are related to cisplatin resistance and are consistent with other studies. Besides, future investigation on AR expression in patients with EMC might be able to differentiate low cisplatin response patients.

Contradictory Results of AR Effects on Cell Stemness

Androgen/AR signal is a confounding factor and contributes to prostate cancer (PCa) initiation, progression, and relapse of therapies. In addition, it is also claimed to participate in various organ developments in terms of stemness, yet inconsistent. Androgen/AR could inhibit normal and cancerous prostate stem cell growth³⁶⁻³⁹ or promote stem/progenitor cells.⁴⁰⁻⁴² In regard of nonprostatic organs, androgen/AR signal exerts a suppressor role in stem/progenitor cells.⁴³⁻⁵¹

We have compared the long-term (5 weeks) antiandrogen Casodex effects on CD133+ populations in the LNCaP cells using the data of this article. We found Casodex promotes PCa stemness that is consistent with the data published by Lee et al (data not show).³⁸ Our data demonstrated that expression of AR in Ishikawa EMC cells could promote CD133+ and S/P cells to mediate cisplatin sensitivity, which is a novel finding.

Figure 3. The AR has little effect on cell growth, yet promotes cell migration and EMT. A, The WST-I assay for Ishi-par and Ishi-AR cells. The OD 590 nm absorbance readings on 0, 2, 4, and 6 days were recorded and plotted on the graph. B, Cell counting assay for Ishi-par and Ishi-AR cells. The cell numbers on 0, 2, 4, and 6 days were recorded and plotted on the graph. The mean values were from 3 independent experiments. C, The AR promotes cell migration in the cisplatin-resistance EMC cells. The Ishi-par and Ishi-AR cells were treated with cisplatin for 14 days and then measured using wound-healing assay. Wound closure area (μm^2) was measured as described in the Methods section. D, The AR promotes EMT in the cisplatin-resistant EMC cells. The Ishi-par and Ishi-AR cells were treated with cisplatin for 14 days and then measured using EMT markers (E-cadherin [E-Cad] and vimentin) expressed by immunoblot assay. The expression of AR was also measured to confirm overexpression of Ishi-AR, while actin expressions were served as loading controls. AR indicates androgen receptor; EMC, endometrial cancer; EMT, epithelial–mesenchymal transition; OD, optical density.

Inconsistency of Androgen/AR Roles in EMC Progression

Majority opinions recognized that androgen/AR signals play positive roles in EMC cancer initiation and progression; however, inconsistent studies exist. Yang et al showed that single nucleotide polymorphisms (SNPs) in *AR* gene has little association with EMC progression and patient status.⁵² Ju et al also demonstrated no correlation of AR CAG lengths in patients with EMC.⁵³ Those inconsistencies may be due to different sampling of patients with EMC, which implicates more sophisticated regulation of androgen/AR in EMC progression, that is, cancer stemness in drug resistance. In our study, we found that androgen/AR signal promotes CSPC that is associated with cisplatin resistance and sensitivity.

This conclusion is consistent with majority theme in EMC field.

Androgen Receptor Expression in EMC and the Potential Association of AR Expression in EMC Chemotherapy Resistance

Because of its lower adverse effects, cisplatin is recognized as the first-line chemotherapy agent for patients with EMC⁵⁴; nonetheless, efficiency remains a major problem.⁴ Therefore, combination therapies of traditional chemotherapy agents with molecular targeting agents are potential regimens for managing patients with EMC.^{55,56} Recent study showed that

Figure 4. The AR enhances CSPC in cisplatin-resistant Ishikawa cells. **A**, The AR mRNA expressions in naive and 7-day cisplatin-treated Ishi-par and Ishi-AR cells. The mRNA expressions were examined by AR-specific primers. β -Actin expressions served as the loading control. The values were calculated by comparing naive Ishi-par cells' AR expression and presented as fold changes. **B**, Cell surface CD133 expressions in cisplatin-resistant Ishi-par and Ishi-AR cells were measured using flow cytometry assay. The par-iso and AR-iso represented the IgG-PE-conjugated isotype antibody stainings of the cells. The par-CD133 and AR-CD133 represented CD133-PE-conjugated antibody staining of cells. The dashed lines (orange dash line: par-CD133 and green dash line: AR-CD133) indicated the mean CD133 fluorescence intensity. **C**, Table shows AR reduces cisplatin cytotoxicity and CSPC characteristics. The naive state Ishi-par and Ishi-AR cells' IC₅₀ against cisplatin were tested. The cisplatin-resistant state (14 days treatment), Ishi-par and Ishi-AR cells IC₅₀, and CD133 population were also measured. AR indicates androgen receptor; CSPC, cancer stem/progenitor cell; mRNA, messenger RNA.

AR overexpression was suspected to be involved in lapatinib (human epidermal growth factor receptor 2 [HER2] and epidermal growth factor receptor [EGFR] kinase inhibitor) resistance and EMT in vitro. Up to date, there is no evidence that endogenous AR expression is associated with EMC chemotherapy resistance or stemness in patients with EMC. However, AR is expressed in normal and malignant endometria,^{57,58} and its role has never been examined in EMC. In the current report, the IC₅₀ in Ishikawa cells is comparable with other reports,^{59,60} which indicates reliable examining system in our study. In addition, adding AR to the cells could increase IC₅₀ against cisplatin treatments. Whether AR has the potential to influence the current combination therapy trials is of great interests.

Genomic and Nongenomic Androgen/AR Actions on CSPC-Related Gene Expression

Our result showed that androgen/AR action upregulates CSPC-related gene expression at differential levels (Figure 4). Using PReMod, we failed to find obvious AR regulatory clusters

within the genome of those genes. The transcriptional binding cluster prediction by PReMod is based on the classical putative ARE, which is located within certain regions that contain several major transcription factor binding sites. As 1 transcriptional factor, androgen/AR action is conventionally thought to bind directly to ARE and regulate gene expression. However, the secondary regulation of androgen/AR actions occupied more than half of gene transcription events upon its impact on the cells.⁶¹ Meanwhile, androgen/AR nongenomic action was proposed to be involved in the normal function of the uterus and in disease progression as well.⁶² We cannot exclude the possibility that androgen/AR directly regulates CSPC-related gene expression through classical transactivation function; yet, need further studies.

Authors' Note

LC and WCC contribute equally to this work. LMC and WCC executed the experiments and composed the manuscript. YCH and YYC provided critical opinions and discussions on the manuscript. BYB, HCH, and WMC executed the flow cytometry experiments and edited

Figure 5. The mRNA expression of CSPC marker genes. A, CSPC-related transcription factors SCF, Bmi-1, Nanog, Oct-4, and Notch mRNAs were measured. The expression levels were calculated by comparing Ishi-AR to Ishi-par cisplatin-resistant cells and presented in fold changes. B, CSPC-related markers CD24, CD133, CD44, ABCG2, CD117, and Nestin mRNAs were measured. C, The AR enhances cisplatin-resistant genes MDR1, ABCB5, and ABCG2 mRNA expressions in endometrial cancer (EMC) cells. The expression levels were calculated by comparing Ishi-AR to Ishi-par cisplatin-resistant cells and presented in fold changes. The mean values were from 3 independent experiments. AR indicates androgen receptor; CSPC, cancer stem/progenitor cell; mRNA, messenger RNA.

the manuscript. CRS provided scientific advises. WLM developed concepts, coordinated the project, and prepared the manuscript.

Supplemental Table 1 is available at <http://rs.sagepub.com/supplemental>.

Declaration of Conflicting Interests

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