Equol inhibits prostate cancer growth through degradation of androgen receptor by S-phase kinase-associated protein 2

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With the introduction of prostate-specific antigen (PSA) screening, the prevalence rate of prostate cancer dramatically increased in Europe and North America in the 1980s, followed by Japan in the 1990s. Accordingly, prostate cancer is the most common non-cutaneous cancer and the second leading cause of cancer-related mortality among the male population in these countries. Owing to widespread PSA screening, the stage at diagnosis has migrated to earlier stages, accompanied by improved cancer-specific survival among PSA-screened populations, as shown in the European Randomized Study of Screening for Prostate Cancer trial undertaken in European countries,1 although a controversial result has been reported from the Prostate, Lung, Colorectal and Ovarian Cancer Screening trial carried out in the USA. However, the latter report suffered from a high contamination rate in the control group, among whom more than 50% underwent PSA screening.2

Thus, because of such a high prevalence rate of prostate cancer and its long latency period, a preventative strategy for prostate cancer appears to attractive, and various agents, including natural and chemical compounds such as vitamin E, selenium, and 5α-reductase, were studied in large-scale clinical trials, but failed to show significant results. A large amount of soy isoflavones are consumed in Asian countries, where the prevalence rate of prostate cancer is comparatively low, which suggests that soy isoflavones may be a possible agent for prostate cancer prevention.3,4 In fact, two randomized clinical trials have examined whether soy proteins and isoflavones can prevent the development of prostate cancer, with both showing a marginal reduction in prostate cancer detection.5–8 A recent publication has clearly revealed the significant preventative value of soy by meta-analysis.9 In addition, therapeutic effects of soy isoflavones have also been expected and examined in clinical trials for prostate cancer, especially localized and low-aggressive cancer.10–13

As for proof of concept, numerous basic research studies have been undertaken to reveal the significance and the mechanism of soy isoflavones on anticarcinogenesis and their anticaner effects. As a result, chemopreventive and therapeutic effects of soy isoflavones have been shown and several mechanisms of their effects, including the disruption of growth signaling, induction of apoptosis, and inhibition of angiogenesis by phytoestrogens and their antioxidant properties have been proposed.3,4 Among them, the inhibitory effect on androgen receptor (AR) signaling in prostate cancer has been considered as one of the major effects of soy isoflavones. Davis et al.14 reported that soy isoflavones suppressed AR as well as PSA expression at the transcription level in prostate cancer cells.
Later, conversely, Basak et al.\(^{15}\) showed that soy isoflavones similarly suppressed AR at the protein level owing to its loss of stability, but not AR at the mRNA level in prostate cancer cells. Thus, there is controversy regarding the mechanisms regulating AR expression by soy isoflavones.

Therefore, in this study, we aimed to further reveal the mechanism regulating AR expression in prostate cancer cells, as well as the differential effect of isoflavones on cell proliferation in prostate cancer cells. Then, we identified the key molecule regulating AR expression by the most potent equal among soy isoflavones, and revealed its expression in prostate cancer.

Materials and Methods

**Cell culture.** Human prostate cancer LNCaP and 22Rv1 cells were obtained from ATCC (Manassas, VA, USA) and authenticated by short tandem repeat analysis. Cells were cultured in RPMI-1640 media (Life Technologies, Carlsbad, CA, USA) supplemented with 10% FBS. LNCaP cells were passaged between 10 and 40 times before use. CxR cells were established and maintained as described previously.\(^{10}\) The cell lines were maintained in a 5% CO\(_2\) atmosphere at 37°C.

**Antibodies and reagents.** Antibodies against AR (sc-816), p27 Ki-67 (sc-528), and ubiquitin (sc-272898) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-p27 Ki-67 (sc-528), and ubiquitin (sc-271289) were purchased from Sigma-Aldrich, CalbioChem (San Diego, CA, USA), respectively. Equol, genistein, and daidzein were purchased from Sigma-Aldrich (St. Louis, MO, USA) and Epitomics (Burlingame, CA, USA), respectively. Exopol, genistein, and daidzein were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and Epitomics (Burlingame, CA, USA), respectively. Equol, genistein, and daidzein were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and Epitomics (Burlingame, CA, USA), respectively. Dihydrotestosterone (DHT), MG132, and cycloheximide were obtained from Sigma-Aldrich (45405, G6776, and D7802).

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**Co-immunoprecipitation assay.** Co-immunoprecipitation assays were carried out as previously described.\(^{17}\) Briefly, whole-cell extracts (500 μg) were incubated for 2 h at 4°C with 1.0 μg anti-AR antibody and p27 antibody with 20 μL protein A/G agarose (Santa Cruz Biotechnology). The immunoprecipitated samples were washed three times, and the immunoprecipitated samples and pre-immunoprecipitated samples (50 μg) as inputs were subjected to Western blot analysis with the indicated antibodies.

**Cytotoxicity analysis.** Cytotoxicity analysis was carried out as described previously.\(^{18-20}\) Briefly, prostate cancer cells (2 × 10\(^4\)) were seeded in 96-well plates. On the following day, cells were cultured with FBS or charcoal-stripped serum (CS) supplemented media with or without 1 nM DHT for 24 h and various concentrations of equol were applied. After 48 h, the surviving cells were stained using the alamarBlue assay (TREK Diagnostic Systems, Cleveland, OH, USA) at 37°C for 180 min. The absorbance of each well was measured using an ARVO MX plate reader (Perkin Elmer, Wallingford, MA, USA). The results are representative of at least three independent experiments.

**Statistical analysis.** The statistical significance of most differences between groups was calculated using Prism 19 software (GraphPad, San Diego, CA, USA). All data were assessed using Student’s t-test. Levels of statistical significance were set at P < 0.05.

**Results**

**Equol potently inhibits AR and PSA expression.** We examined which soy isoflavones, including equol, genistein, and daidzein, were most potent for suppressing AR signaling. As shown in Figure 1(a), LNCaP cells showed impairment of AR at the protein level owing to its loss of stability, but not AR at the mRNA level in prostate cancer cells. Thus, there is controversy regarding the mechanisms regulating AR expression by soy isoflavones. Consistently, expression of the major AR-target gene PSA, was also reduced by soy isoflavones. However, AR mRNA expression was little affected by soy isoflavones. In contrast, PSA mRNA expression was significantly inhibited by soy isoflavones. Equol, suggesting non-transcriptional regulation of AR expression by equol. Interestingly, equol, which is a major metabolite of daidzein, most potently inhibited AR and PSA expression compared with genistein and daidzein. Therefore, we used equol in subsequent experiments.

**Androgen receptor protein and cell proliferation suppressed by equol with or without androgens.** To examine the effect of androgens on AR degradation by equol, LNCaP cells were incubated in CSS-supplemented media with or without DHT and treated with equol. When LNCaP cells were treated with equol, the AR protein level decreased with or without DHT (Fig. 2a). Consistently, PSA mRNA levels were also decreased by equol with or without DHT, although equol repeatedly failed to affect AR mRNA levels (Fig. 2b). Cell proliferation was also retarded by equol with or without DHT (Fig. 2c).
The inhibitory effects of equol on AR activity and cell proliferation were intriguingly prominent in DHT treated cells compared with DHT untreated cells (Fig. 2), suggesting that the effect of equol is partially androgen-dependent.

**Effect of equol on AR expression in CxR and 22Rv1 cells.** We analyzed the effect of equol in CxR cells, which are castration-resistant derivatives of LNCaP cells. Interestingly, equol induced downregulated AR expression at the protein level (Fig. 3a), but not at the mRNA level (Fig. 3b). Accordingly, PSA expression was reduced by equol at both mRNA and protein levels (Fig. 3a,b), although the inhibitory effects on AR and PSA suppression by equol was less prominent compared with that in LNCaP cells (Fig. 1b). However, in another castration-resistant cell line, 22Rv1, full-length AR as well as AR variant (AR V7) expression was little affected by equol at both mRNA and protein levels (Fig. 3c,d).

**Equol reduces cell viability most prominently in LNCaP cells, compared with CxR and 22Rv1 cells.** Subsequently, we examined cytotoxicity by equol in LNCaP, CxR, and 22Rv1 cells. As shown in Figure 4(a), LNCaP cells showed prominent susceptibility to equol. However, equol had little or no effect in CxR and 22Rv1 cells, respectively. The cleaved form of poly(ADP-ribose) polymerase, indicating an induction of cellular apoptosis, increased in LNCaP cells but not in CxR or 22Rv1 cells in the presence of equol (Fig. 4b).

**Equol suppresses AR expression through AR degradation.** To investigate the mechanism of AR repression by equol, LNCaP cells were treated with cycloheximide, inhibiting de novo protein synthesis, or MG132, inhibiting proteasome degradation. As shown in Figure 5, although equol alone induced AR protein degradation, MG132 blocked AR degradation by equol. However, cycloheximide reversed the AR level changes by equol, indicating that equol suppresses AR expression through proteasome degradation. Intriguingly, MG132 treatment induced a truncated form of AR, especially with equol (Fig. 5), as previously reported.(21)

**Androgen receptor degraded by equol through interaction between AR and Skp2.** It has been reported that Skp2 regulates AR expression through ubiquitin-mediated degradation. To assess the role of Skp2 on AR expression after treatment of equol, we transfected Skp2-specific siRNA into LNCaP cells and then examined AR expression and cell viability. In the presence of equol, knockdown of Skp2 resulted in restored AR expression, suggesting that Skp2 is involved in the proteasome degradation of AR.
expression, which was accompanied by PSA induction, and overcame cell growth inhibition compared with control siRNA (Fig. 6a,b). These results suggest that equol induced AR degradation through Skp2. Next, we examined whether equol treatment affects the interaction between AR and Skp2. Interestingly, the interaction between AR and Skp2 was increased in the presence of equol, even though the number of non-immunoprecipitated Skp2 and AR proteins was decreased by equol (Fig. 6c).

Because Skp2 is one of ubiquitin ligase to regulate tumor suppressor genes, including CDK inhibitor p27, we tested the hypothesis that p27 degradation is suppressed during AR degradation. In LNCaP cells, equol treatment resulted in p27 accumulation (Fig. 6d). Then, we tested the ubiquitination of p27 in LNCaP cells after treatment with equol and found that the ubiquitination of p27 was suppressed.

Expression of Skp2 in prostate cancer cell lines. Skp2 expression was important to suppress AR expression in the presence of equol. We found the effect of equol was different between cell lines (Fig. 4). Then, we examined Skp2 expression levels in prostate cancer cell lines. As shown in Figure 7, Skp2 expression levels were decreased in LNCaP, CxR, and 22Rv1 cells, in descending order, at both mRNA and protein levels.

Discussion

Soy isoflavones consist of several types of components, such as more abundant daidzein and genistein and less abundant glycitin, as well as equol as a metabolizer of daidzein. Previously, equol has been shown to be more active compared with other soy isoflavones. In line with previous studies, this study showed that equol exerted the most powerful effect to suppress AR expression in LNCaP cells compared with non-metabolized soy isoflavones, such as daidzein and genistein, suggesting a crucial role metabolizing daidzein into equol by intestinal flora, as suggested by Akaza. Previously, there has been some controversy regarding AR regulation by soy isoflavones. The present study revealed that AR protein expression by equol was regulated by the proteasomal pathway, but not through transcriptional or translational mechanisms, which supports the results of Basak et al. Itsumi et al. showed that histone deacetylase inhibition by genistein leads to the hyperacetylation of the AR-stabilizing chaperone heat shock protein 90, which underlines the chaperone function and accelerates ubiquitination and degradation of client proteins including AR. Similarly, Li et al. reported that AR activity was regulated by isoflavones through Akt/Foxo3a/Akt/Foxo3b phosphorylation pathway. In our study, we showed that equol treatment downregulated Skp2, which is a ubiquitin ligase for AR, leading to AR degradation. This finding suggests that equol may act as a potential therapeutic agent for prostate cancer treatment.
glycogen synthase kinase-3β signaling, resulting in inhibition of AR translocation into the nucleus, thereby promoting AR degradation. For AR ubiquitination, the cascade of ubiquitin ligases from E1 to E3 plays a crucial role. Among these proteins, several E3 ubiquitin ligases have been reported to modulate AR function. Previous studies have shown that the MDM2(29) and Skp2(22) E3 ligases promote AR depletion and inhibit AR function, whereas RNF6(30) and Siah2(31) E3 ligases play opposite roles. Because MDM2 has been shown to be downregulated by the soy isoflavone genistein,(32) suggesting inconsistent effects of soy isoflavone on AR and MDM2, we focused on Skp2 as a possible mediator of AR degradation by the soy isoflavone equol. Next, we successfully revealed the functional role of Skp2 that promoted the interaction with AR and augmented AR degradation in the presence of equol. Previously, soy isoflavones have been shown to bind to the AR ligand-binding domain and act as an anti-androgen. In addition, AR mutation (T877A) in LNCaP cells might influence the effect of soy isoflavones, as it has been known that AR mutation can affect ligand specificity. These reports suggest the possibility that these functions also influence the growth inhibition in equol-treated LNCaP cells. Consistently, our study has shown that equol inhibited AR function as well as cell proliferation in androgen-dependent LNCaP cells in a partially androgen-dependent manner.

As a member of the F-box protein family, Skp2 is responsible for the degradation of several tumor suppressors, such as p27(38) and Foxo1. We showed that equol induced accumulation of p27. These results suggest that expression of tumor
suppressor genes including p27 was maintained through equol promoting AR degradation by Skp2.

In addition, this study has shown that Skp2 expression was decreased in castration-resistant CxR and 22Rv1 cells, compared with androgen-dependent LNCaP cells. This decrease in Skp2 would bring about decreased AR degradation and thereby increased AR expression. The increased AR degradation (40), as well as augmented AR expression by various mechanisms (41) are well known to be major causes for obtaining castration resistance in prostate cancer. Therefore, decreased expression of Skp2 may be one cause of castration resistance in castration-resistant CxR and 22Rv1 cells.

In this study, we showed that 50 μM equol effectively suppressed AR expression and cell growth. This concentration is higher than ordinary serum and prostate tissue concentrations of equol and isoflavones (42–43). Therefore, to obtain high concentration levels of equol in prostate tissue, the development of methods to increase equol bioavailability by increasing the absorption from dietary intake and conversion into equol would be required, as suggested by Akaza. (26)

Taken together, this study has provided proof of concept to use soy isoflavones, especially equol, for chemoprevention and therapeutics of prostate cancer based on the mechanism that equol augmented Skp2-mediated AR degradation. Moreover, Skp2 expression is crucial for the effect of soy isoflavones on AR expression as well as prostate cancer proliferation, suggesting that soy isoflavones may be applicable for precancerous and cancerous prostates expressing abundant Skp2.

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Disclosure Statement

The authors have no conflict of interest.
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