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| 項目     | 博士論文本文 以下に掲載：大筆 信夫, 等 \textit{金沢大学学術情報リポジトリ} 共著者：
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Abstract. Background: Prevention of the development of castration-resistant prostate cancer is an important issue in maintaining the quality of life of the patients. We explored the effect of 2'-hydroxyflavanone on proliferation and androgen responsiveness using prostate cancer cell lines. Materials and Methods: To investigate the effect of 2'-hydroxyflavanone on proliferation, prostate cancer cells were treated with 2'-hydroxyflavanone. Androgen-responsiveness in LNCaP cells was confirmed by luciferase assay after transfection of luciferase reporter driven by prostate specific antigen promoter. To detect androgen receptor (AR) expression, reverse transcriptase polymerase chain reaction and western blot analysis were conducted. Results: 2'-Hydroxyflavanone inhibited the proliferation of PC-3 and DU145 cells by induction of apoptosis. 2'-Hydroxyflavanone inhibited the proliferation of LNCaP cells stimulated by androgens and attenuated androgen-responsiveness through down-regulation of AR protein. Conclusion: 2'-Hydroxyflavanone not only inhibited proliferation of prostate cancer cells, but also repressed androgen-responsiveness, suggesting that it might be an useful agent in preventing recurrence of prostate cancer.

Since the androgen receptor (AR) axis is the main route for development and progression of prostate cancer (PCa), androgen-deprivation therapy (ADT) is conducted as a first-line hormonal therapy using medical castration, such as luteinizing hormone releasing hormone (LH-RH) agonists, LH-RH antagonist, and antiandrogen for advanced PCa. After an initial response to ADT, however, PCa eventually loses responsiveness to ADT and progresses into what is termed castration-resistant PCa (CRPC).

The AR axis also plays an important part in the process when hormone-sensitive PCa become CRPC. Although serum testosterone decreases to less than 5% before starting ADT, PCa adapts to low serum testosterone level by several mechanisms. One other important factor is the adrenal androgen, dehydroepiandrostenedione (DHEA). DHEA is metabolized into testosterone, and then converted to dihydrotestosterone (DHT) by 5α-reductase in PCa tissue, which then activates the AR. In fact, the concentration of DHT in PCa tissue remains 20 to 40% of pretreatment values (1-3). Interaction of epithelial and stromal cells plays an important role in the production of DHT in PCa tissue. After castration, adrenal androgen DHEA is metabolized into DHT in stromal cells and epithelial cells coordinately (4).

Moreover, CYP17A inhibitors, abiraterone acetate and TAK-700, which inhibit conversion from pregnenolone into DHEA, is effective for more than 70% of CRPC after docetaxel-treatment. These results indicate that the AR axis affects the recurrence of PCa in patients resistant to docetaxel.

Important enzyme in intratumoral androgen synthesis mediating through interaction of epithelial and stromal cells are type 3 and type 5 17β-hydroxysteroid dehydrogenase (HSD17B3 and HSD17B5) and 3β-hydroxysteroid dehydrogenase (3β-HSD). In particular, HSD17B3 catalyzes the formation of testosterone from 4-androstenedione (adione) in the testis and peripheral tissues (5). However, the function of the testes is lost in PCa after ADT, the main androgen synthesis enzyme in CRPC is HSD17B5. It is known that AKR1C3 aldo-keto reductase acts as a HSD17B5 (6, 7).
AKR1C3 was found to be up-regulated in patients with PCa, especially, in those with metastatic PCa and CRPC (8-11). Moreover, overexpression of AKR1C3 promoted PCa proliferation (12). These findings suggest that hyperactivation of AKR1C3 might affect the recurrence of PCa.

Flavonoids are a large group of polyphenolic compounds present in foods and beverages of plant origin, and are subdivided into six subclasses: flavonols, flavones, flavanones, flavan-3-ols, anthocyanidins, and isoflavones (13). Flavonoids display a broad range of pharmacological activity, such as antioxidative, anti-inflammatory, and antiproliferative activities (13-15). Flavonoids have been shown to inhibit AKR1C3 activity in vitro (16, 17). 2'-Hydroxyflavanone (2’HF), which is a flavanones in particular had a strong inhibitory effect on AKR1C3 in vitro (16).

In the present study, we investigated the effect of 2’HF on proliferation and androgen responsiveness using PCa cell lines.

Materials and Methods

Cell lines and cell proliferation assay. LNCaP and DU145 cells (ATCC, Manassas, VA, USA) were cultured in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 1% penicillin/streptomycin (P/S; Invitrogen, Carlsbad, CA, USA) and 5% fetal bovine serum (FBS; Sigma–Aldrich, St. Louis, MO, USA), respectively. PC-3 cells (ATCC) were cultured in RPMI -1640 supplemented with 1% P/S (Invitrogen) and 5% FBS. Twenty-four hours after plating at a density of 5×10⁴ cells onto 12-well plates with DMEM-5% CCS, LNCaP and PC-3 cells were transfected using Lipofectamine transfection reaction (Invitrogen) with 0.5 μg of luciferase reporter plasmid, pGL3PSA-5.8, driven by a 5.8 kb PSA promoter (20). Twenty-four hours after transfection, cells were treated by the addition of DHT with and without 2’HF for 24 hours. After treated cells were harvested, cells were lysed in luciferase lysis buffer (Promega, Madison, WI, USA) and the luciferase activity was quantitated by a luminometer. For overexpression of EGFP-fAR in LNCaP cells, 5×10⁴ LNCaP cells were co-transfected with 0.1 μg of pEGFP-fAR and 0.4 μg of pGL3PSA-5.8, and then cells were further treated with adione, DHT and/or 2’HF for 24 hours.

Apoptosis assay. To investigate whether 2’HF causes PCa cells to undergo apoptosis, the Annexin-V-FLUOS Staining kit (Roche, Mannheim, Germany) was used according to the manufacturer’s protocol. In brief, 1×10⁴ LNCaP, and DU145 cells, and 5×10⁴ PC-3 cells were seeded in 6-well plates with DMEM-5% CCS. They were changed every two days. In each experiment, cells were harvested and the numbers of the cells were counted in triplicate using a hemocytometer. The data shown represent the means±SD of three replicates.

Reverse transcriptase polymerase chain reaction (RT-PCR) and western blot analysis. For RT-PCR, 24 hours after plating at a density of 1×10⁵ cells onto 6-well plates with DMEM-5% CCS, cells were treated with or without Adione, DHT and/or 2’HF for 24 hours and total RNA was extracted. Total RNA extraction from cells and RT-PCR for AR, prostate specific antigen (PSA), and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was performed as described previously (2, 4).

For western blot analysis, total protein was extracted from cells as described previously (18). Protein was quantified according to the method of Bradford, and equal amounts of protein were electrophoresed on a 10% or 12.5% Ready Gel J (Bio-Rad, Hercules, CA, USA). Membranes were incubated with mouse monoclonal antibody against AR (NH27) (19) and GAPDH (Novus Biologicals, Littleton, CO, USA). Horseradish peroxidase-conjugated secondary antibody against mouse monoclonal or rabbit monoclonal antibody was used and protein bands were visualized and quantitated with chemiluminescent reagent (SuperSignal West Pico Chemiluminescent Substrate; Pierce, Rockford, IL, USA) and ChemiDoc XRS (Bio-Rad).

Recombinant plasmid constructs. Recombinant plasmid pEGFP-fAR that expresses full-length wild-type AR fused with green fluorescent protein (GFP) was constructed by inserting the full-length AR cDNA of pSGAR2, which is driven by SV40 promoter (19) at -24 to 3110 bp of start codon, into pEGFP-C1 (Invitrogen, CA, USA). The insert configurations of fAR cDNAs were confirmed by sequence analysis.

Luciferase assay. To evaluate AR transcriptional activity, 24 hours after plating 5×10⁴ cells on 12-well plates in DMEM-5% CCS, LNCaP and PC-3 cells were transfected using Lipofectamine transfection reaction (Invitrogen) with 0.5 μg of luciferase reporter plasmid, pGL3PSA-5.8, driven by a 5.8 kb PSA promoter (20). Twenty-four hours after transfection, cells were treated by the addition of DHT with and without 2’HF for 24 hours. After treated cells were harvested, cells were lysed in luciferase lysis buffer (Promega, Madison, WI, USA) and the luciferase activity was quantitated by a luminometer. For overexpression of EGFP-fAR in LNCaP cells, 5×10⁴ LNCaP cells were co-transfected with 0.1 μg of pEGFP-fAR and 0.4 μg of pGL3PSA-5.8, and then cells were further treated with adione, DHT and/or 2’HF for 24 hours.

Liquid chromatography mass spectrometry/mass spectrometry (LC-MS/MS). After plating 5×10⁴ cells on 12-well plates in DMEM-5% CCS, PC-3 cells were treated with 10 nM adione or 10 nM testosterone in the absence and presence of 10 μM 2’HF. Then the media were collected 24 hours later. The concentration of adione, testosterone, and DHT in media was measured by LC-MS/MS (Division of Pharmacological Research, Aska Pharma Medical Co. Ltd., Kawasaki, Japan).

Visualization of AR localization. Twenty-four hours after transfection of pEGFP-fAR into PC-3 cells, cells were treated with or without 10 μM 2’HF for 24 hours. Consequently cells were cultured in the absence or presence of 10 nM DHT for 8 hours, and fAR fused to green fluorescent protein (GFP) was visualized by FSX100.

Statistical analysis. Statistical significance was determined by using Prism 6.0 software, the χ² test was utilized to assess the significance between different proportions. Analysis of continuous variables between different groups was assessed by one-way analysis of variance followed by Fisher’s protected least significant difference test. *, **, and *** in Figure represent significant difference p<0.05, p<0.01, and p<0.001, respectively.

Results

Effect of 2’HF on PCa cell proliferation. In order to investigate the effect on PCa cell proliferation, androgen-
In order to further investigate the effect of 2'HF, we transfected LNCaP cells with an AR expression plasmid vector (pSGAR2) and examined the effect of 2'HF (Figure 2B). PSA promoter activity induced by 10 nM adione and 1 nM testosterone in the presence of wild-type AR was repressed by 10 μM 2'HF, suggesting that 2'HF might affect AR activity directly independently of androgen concentration in the medium.

2'HF does not affect HSD17B and 5α-reductase activity in PCa cells. Since 2'HF repressed AR activity induced by testosterone and DHT, as well as adione, we determined if 2'HF affected the androgen concentration in medium from cell cultures. We treated PC-3 cells with 10 nM adione or 10 nM testosterone in the absence and presence of 10 μM 2'HF and measured the concentration of adione, testosterone, and DHT in the medium 24 hours later by Liquid chromatography mass spectrometry/mass spectrometry (LC-MS/MS). 2'HF did not change testosterone and DHT concentration after addition of adione, and did not change DHT concentration after the addition of testosterone, suggesting that 2'HF does not affect HSD17B and 5α-reductase activity in vitro (Figure 3).

2'HF inhibited the expression of AR protein but not AR mRNA. To reveal the mechanism of 2'HF repression AR activity, we first investigated the expression level of AR mRNA in LNCaP cells. Although 2'HF repressed the expression of PSA and other androgen-responsive genes induced by adione, testosterone, and DHT, the expression of AR mRNA did not change regardless of the presence or absence of 2'HF (Figure 4A). Next we investigated whether 2'HF affects the expression of AR protein. Western blot analysis revealed 2'HF down-regulated the expression of AR protein in LNCaP cells in a dose-dependent manner (Figure 4B). In addition, the level of exogenous AR in PC-3 cells transfected with pSGAR2 driven by SV40 promoter was also repressed by 2'HF.

Nuclear localization of AR. AR is usually localized in the cytoplasm in the absence of androgen and is translocated into the nucleus in the presence of androgen. We determined whether 2'HF affects AR localization. We transfected PC-3 cells with pEFGP-fAR plasmid that express full-length AR fused to GFP protein and recorded AR localization in the cells with pEGFP-fAR plasmid that express full-length AR. Western blot analysis revealed 2'HF down-regulated the expression of AR protein in LNCaP cells in a dose-dependent manner (Figure 4B). In addition, the level of exogenous AR in PC-3 cells transfected with pSGAR2 driven by SV40 promoter was also repressed by 2'HF.

Discussion

Once androgen-sensitive PCa becomes CRPC during first-line hormonal therapy, physicians often conduct second-line therapy. PCa cells.
Figure 1. Effect of 2’HF on the proliferation of PC-3, DU145, and LNCaP cells. A: Twenty-four hours after 5×10⁴ PC-3 or DU145 cells were plated, cells were treated with the indicated concentrations of 2’HF, then cells were counted. The media were changed every two days. B: After 5×10⁴ PC-3 or DU145 cells were plated on 6-well plates, cells were treated for 72 hours with or without 10 μM 2’HF, they were incubated with 100 μl Annexin-V-FLUOS labeling solution. They were analysed by phase contrast (left) and fluorescence microscopy (right). C: After LNCaP cells were treated with 0, 10 nM 4-androstenedione (adione), 1 nM testosterone (T), or 1 nM dihydrotestosterone (DHT) in the presence of the indicated concentration of 2’HF for four days, the numbers of LNCaP cells were counted. D: LNCaP cells 1×10⁵ were plated onto 6-well plates. Seventy-two hours after treatment with or without 10 μM 2’HF, they were incubated with 100 μl Annexin-V-FLUOS labeling solution. They were analyzed by phase contrast (left) and fluorescence (right) microscopy. E: Twenty-four hours after seeding of LNCaP cells, cells were treated with 10 nM Adione or 10 nM DHT in the presence of indicated concentrations of 2’HF for 20 hours. The expression level of PSA mRNA was determined by RT-PCR. *p<0.05, ***p<0.001.
hormonal therapy and sequentially conduct chemotherapy using docetaxel. Although docetaxel is effective for 70% of CRPC, CRPC eventually shifts to become docetaxel-resistant. In such a situation, the prognosis of the patients is poor. Therefore, it is extremely important to prevent PCa from recurrence during hormonal therapy, without severe side-effects.

Flavonoids have antitumor activity, inducing apoptosis via tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), nuclear transcription factor-κB (NF-κB) reactive oxygen species, and peroxisome proliferator-activated receptor-gamma (PPARγ)-dependent, and independent pathways (21-24). Recently it was reported that among flavonones, 2’HF showed the most potent tumor inhibitory activity by stimulating caspase-mediated apoptosis of colon cancer cells (25). This apoptosis by 2’HF was associated with up-regulation of nonsteroidal anti-inflammatory drug-activated gene 1 (NAG-1) expression through induction of

Figure 2. Effect of 2’HF on androgen receptor (AR) activity in prostate cancer cells. A: Twenty-four hours after transfection of LNCaP cells with luciferase reporter plasmid (pGL3PSAp-5.8), cells were treated with 0, 10 nM 4-androstenedione (adione), 1 nM testosterone (T), or 1 nM dihydrotestosterone (DHT) in the presence of the indicated concentrations of 2’HF for 24 hours, luciferase activity was then examined. B: Twenty-four hours after transfection of PC-3 cells with 300 ng pGL3PSAp-5.8 and 200 ng pSGAR2, cells were treated with 0, 10 nM adione, or 1 nM T in the absence or presence of 10 μM 2’HF for 24 hours, then luciferase activity was examined.

Figure 3. Androgen biosynthesis from adione or testosterone in PC-3 cells. Twenty-four hours after starting culture of 5×10⁴ PC-3 cells, 10 nM adione or 10 nM testosterone was added to the medium. Aliquots of medium were collected after 24 hours for measuring the concentration of adione, testosterone and testosterone (T).
EGR-1. 2′HF also inhibited cell cycle progression and angiogenesis by reducing vascular endothelial growth factor expression in von Hippel-Lindau (VHL)-mutant renal cell carcinoma (26). In the present study, 2′HF inhibited proliferation of androgen-independent PCa cells via apoptosis at least. We are now investigating the mechanism of apoptosis induced by 2′HF in PCa cells.

2′HF was also a candidate for repression of AKR1C3 activity that stimulates biosynthesis of testosterone from adione (16). Contrary to our expectation, however, 2′HF did not have an inhibitory effect on AKR1C3 activity in cultured PCa cells. Instead, 2′HF repressed testosterone and DHT-induced androgen responsiveness. In the present study, 2′HF was shown, to our knowledge for the first time, to downregulate AR activity. This effect was mediated through downregulation of AR protein expression at least. We also previously confirmed whether other candidates of dietary flavonoids AKR1C3 inhibitors could affect AR activity. One such candidate, naringenin, which is a strong AKR1C3 inhibitor, also repressed AR activity in the presence of DHT, as well as adione (data not shown) (16). Naringenin may also repress AR activity similarly to 2′HF without mediating through AKR1C3. Moreover, 2′HF also inhibited AR translocation into the nucleus. However, it is not clear whether this inhibition is a result of inhibition of the nuclear localization signal or of diminished AR expression. We accept that 2′HF may inhibit AKR1C3 activity and inhibit androgen synthesis from adione to testosterone. A higher concentration of 2′HF may inhibit AKR1C3 activity in PCa cells.

Several mechanisms by which androgen-naive PCa changes to CRPC have been proposed, such as the existence of hypersensitive AR, promiscuous AR, outlaw AR, bypass AR, and alternative spliced truncated AR (27, 28). Existence of adrenal androgen also affects the mechanisms by which PCa becomes CRPC (29). Moreover, intratumoral androgen synthesis, especially, in PCa-derived stromal cells and bone-derived stromal cells, also play an important role in activation of adrenal androgen (4, 30). Therefore, some strategies to block the androgen AR axis have been considered (31). These include: (i) Inhibition of androgen synthesis enzyme upstream of DHEA synthesis in adrenal gland, such as abiraterone acetate and TAK-700 (32, 33); (ii) use of more potent antiandrogen agents, or inhibition of nuclear translocation of AR, such as enzalutamide (34); (iii) inhibition of androgen synthesis from DHEA in tumor cells or stromal cells, e.g. using 5α-reductase inhibitors (35). (iv) diminishing the AR level in PCa cells. Clinical evidence that enzalutamide, abiraterone acetate, and TAK-700 are very effective for CRPC even after docetaxel treatment have proven that the androgen AR axis plays an extremely important role in the transformation of PCa to CRPC (29, 36). However, strong inhibitors of androgen synthesis enzymes such as HSD17B (AKR1C3) or HSD3B, in tumor cells have not yet been identified. Moreover, it may be difficult to overcome the progression of CRPC via the androgen AR axis completely by inhibition of androgen synthesis. Androgen-independent AR activation is also indicated in the progression of CRPC via interleukin-6 (IL-6) activation of signal transducer and activator of transcription 3 (STAT3) (37, 38), and via IL-8 signaling tyrosine kinases Src and focal adhesion kinase (FAK) (39, 40). If androgen-independent AR activation by cytokines results in emergence of CRPC, the inhibition of androgen synthesis would not be effective for these patients. In such cases, repression of AR expression by 2′HF might be effective for inhibition of the AR axis.

Recently, ASC-J9 was synthesized, which stimulates AR degradation-repressed androgen responsiveness (41).
Curcumin, which is a major natural yellow pigment in turmeric and is widely used as a spice and coloring agent in several foods such as curry, has also been shown to down-regulate AR protein (42). We revealed that 2’HF similarly represses AR activity by diminishing AR protein in PCa cells. We will investigate whether 2’HF affects AR ubiquitination and degradation of AR or affects AR translation.

When advanced PCa changes to CRPC during hormonal therapy, physicians often conduct docetaxel-based chemotherapy, which can extend the survival period of the patients (43). However, the effective period is limited because of further progression of CRPC. Overcoming docetaxel resistance, therefore, is important to improve the prognosis of patients. One of mechanisms of taxane resistance is up-regulation of P-glycoprotein expression (44). Polymethoxyflavones, which are components of orange juice, inhibit P-glycoprotein-mediated efflux (45). Since flavonoids have a variety of functions for pharmacological activity, 2’HF may also exhibit inhibitory activity on P-glycoprotein and increase the sensitivity to docetaxel. As a result, 2’HF might reduce adverse effects as well as overcoming docetaxel resistance.

In conclusion, we revealed that the natural product, 2’HF, found naturally in fruits and vegetables, can inhibit not only the proliferation of PCa cells but also androgen responsiveness via down-regulation of AR protein. If we can prevent progression of PCa during ADT by taking natural products such as 2’HF effectively, patients will be able to benefit physiologically and economically. Further analyses are needed to reveal the full range of pharmacological activities of 2’HF.

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