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MiR-8080 recruitment inhibits expression of androgen receptor splicing variants in castration-resistant prostate cancer

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Introduction

Prostate cancer is major leading cause of death in western world. And elucidating the mechanisms of metastasis in castration resistant prostate cancer (CRPC), particularly to the bone, is a major issue for treatment of this malignancy. Reactive oxygen species (ROS) have been identified as important chemical mediators in cell growth and differentiation, Therefore, dietary intake of affecting ROS is effective intervention for prevention and therapy in prostate cancer. As we previously reported, such antioxidants prevents prostate carcinogenesis using newly established CRPC animal model (as described below), and Luteolin (3′, 4′, 5, 7-tetrahydroxy flavone) has anti-oxidative and anti-tumor ability.

Nowadays, enzalutamide was approved for the anti-CRPC drugs, however, spliced variants of androgen receptor (AR) protein such as AR variant 7 (AR-V7), that lack a functional ligand binding domain represented important mechanism of resistance in CRPC to enzalutamide.

In this study, we investigated the anti-oxidative anti-tumor effect of luteolin using our established model. And, we have detected the inhibitory effect of luteolin with the regulation of AR-V7. In addition, we newly identified the regulatory mechanisms of AR-V7 expression via the expression of miR-8080 using miRNA microarray.

Material and methods

(I) Therapeutic effect of luteolin on rat CRPC cell line, PCai1, *in vitro* and *ex vivo*.

Luteolin was treated in PCai1 cells and Pcai1 xenograft model, and mRNA and protein expression was analyzed using qRT-PCR and western blotting. For the evaluation of ROS, DCFH assay was performed. In addition, *flowcytometry in vitro*, and TUNEL assay for the evaluation of apoptosis *in vivo*, were made.

(II) Suppressive effect of luteolin on cell proliferation by induction of caspase-dependent apoptosis via regulation of AR-V7 expression in human CRPC cell lines *in vitro*.

Luteolin was treated in 22Rv1 cells, and mRNA and protein expression was analyzed using qRT-PCR and western blotting. ROS analysis and Gene expression analysis including AR-FL, AR-Vs expression was compared between 22Rv1 with or without 25 μM luteolin.

(III) Therapeutic effect of luteolin on CRPC tumor growth by suppression of AR-V7 in a 22Rv1 xenograft model.

22Rv1 cells were subcutaneously implanted in castrated nude mice. A total of 40 mice were randomly divided into the control and 100ppm luteolin were given by diet. Protein expression analysis of AR-V7 was made.

(IV) Luteolin inhibits AR-V7 by induction of miR-8080 in 22Rv1 cells.

Luteolin was treated in 22Rv1 cells, and mRNA and protein expression was analyzed using qRT-PCR and western blotting. Gene expression analysis was performed using Human Oligo chip 25k. MiRNA expression was compared between 22Rv1 with or without 25 μM luteolin for 48h, and MiR-8080 was newly identified in down regulated miRNA after luteolin treatment. MiR-8080 expression vector and the empty vector (pCMV-MIR) were purchased. MiR-8080 inhibitor and miRNA negative control (NC) were synthesized. MiR-8080 expression vector or inhibitor was transfected into 22Rv1 cells using Lipofectamine 3000. In addition, the combination effect of luteolin and enzalutamide were analyzed using 22Rv1 cells with protein expression analysis of AR-V7.

(V) MiR-8080 enhances the chemotherapeutic effect of enzalutamide in 22Rv1 cells both *in vitro* and *ex vivo*.

Forty eight xenograft tumor of 22Rv1 were prepared. Vehicle, 10mg/kg/day enzalutamide, 100ppm luteolin, and enzalutamide plus luteolin were treated. MiR-8080 and AR-V7 expression were analyzed using qRT-PCR and western blotting.

Discussion

In this study, a dietary intake of luteolin clearly suppressed the tumor growth of CRPC through the induction of apoptosis without any adverse effects. This is the first report to show an effect of dietary luteolin on prostate carcinogenesis and CRPC. Given that luteolin is used as a chemopreventive agent for humans, an oral intake may be a more natural route for administration.

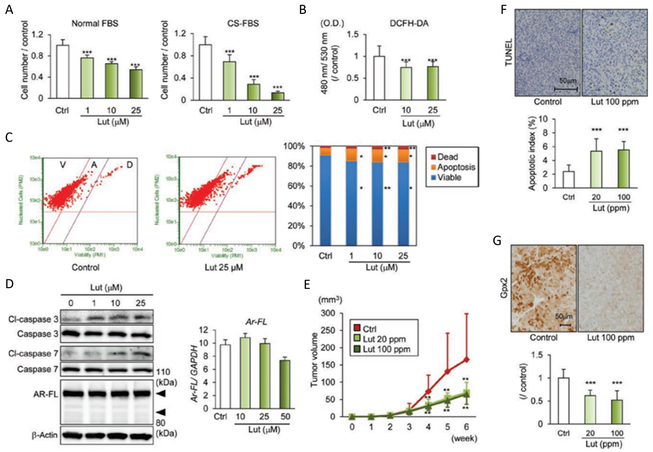
ROS accumulation can induce gene mutations, alterations of the mitochondrial metabolic pathway, and the activation of AR, resulting in the dysregulation of apoptosis and development of prostate cancer. Our data indicate that dietary luteolin suppressed ROS accumulation and induced apoptosis in both TRAP prostate tumors and CRPC tumors of both rat and humans. These findings are consistent with previous reports and indicate that enhancement of apoptosis by protection against ROS is one of the important mechanisms for luteolin as an anti-cancer agent. An anti-oxidative effect and increased apoptosis were also observed in CRPC.

In this study, miR-8080 was identified as being up-regulated by luteolin; it could directly interact with AR-V7 but not with AR-FL. In addition, luteolin also induced miRNAs that targeted AR-FL and decreased AR-FL protein expression. Silencing of AR-V7 by specific siRNA induced decreased proliferation in 22Rv1 cells. AR knock-down also induced growth inhibition in 22Rv1 cells, though the effect was less than for AR-V7. These results suggest that the efficacy of miR-8080/AR-V7 therapy against CRPC may be enhanced by a combination of miRNAs targeting AR-FL.

Most patients are resistant to the drugs including enzalutamide owing to the expression of active AR-Vs, such as AR-V7, in the absence of androgen. AR-V7 is a key protein to consider in any novel strategy of CRPC therapy. The present study indicates that luteolin and its induced expression of miR-8080 improved the chemotherapeutic resistance of enzalutamide against CRPC, both *in vitro* and *in vivo*. These results suggest that a supplemental miR-8080 can be beneficial to the efficacy of enzalutamide.

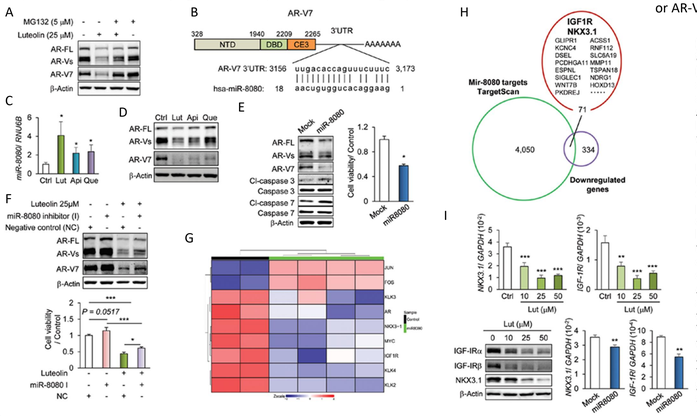
In conclusion, luteolin suppresses both the early stage of prostate carcinogenesis and CRPC via the induction of apoptosis. MiR-8080 induced by luteolin supplementation has an important role in the reduction of AR-V7 protein, resulting in inhibiting tumorigenesis and the enzalutamide resistance of CRPC. Therefore, miR-8080 may be a novel therapeutic target for CRPC.

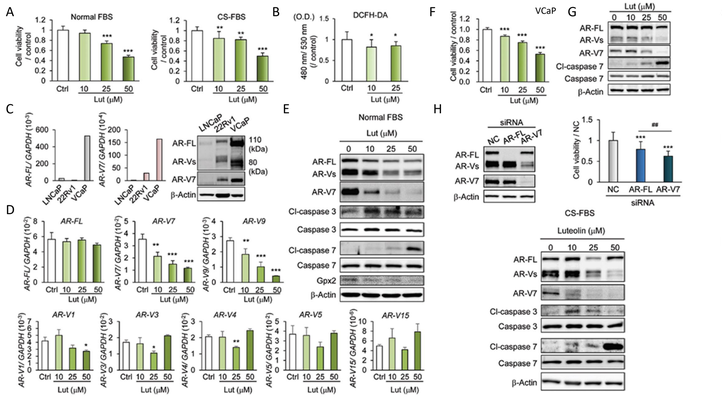
Results



(I) Therapeutic effect of luteolin on rat CRPC cell line, PCai1, *in vitro* and *ex vivo*.

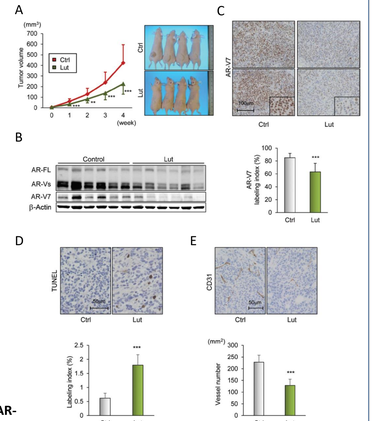
(A) The proliferation rate of PCai1 cells at five days following luteolin (Lut) treatment with normal FBS (left) and CS–FBS medium (right). (B) Intracellular ROS level in PCai1 cells by DCFH-DA assay. (C) PCai1 cells treated with luteolin for 48 h and stained with Guava® ViaCount to separate viable (V), apoptotic (A) or dead (D) cells. The survival status after each treatment is presented in the bar chart. (D) Western blotting analyses for caspases 3 and 7, and cl-caspases 3 and 7, or β-actin in PCai1 cells. (E) Tumor volume of PCai1 xenografts (1.0×10^6) in castrated nude mice. (F–G) Representative TUNEL (F), Gpx2 (G) by immunohistochemistry. $^*P <$ 0.05, $^{**}P <$ 0.001 statistically significant compared with control group.





(II) Suppressive effect of luteolin on cell proliferation by induction of caspase-dependent apoptosis via regulation of AR-V7 expression in human CRPC cell lines *in vitro*.

(A) The proliferation rate of 22Rv1 cells after luteolin (Lut) treatment for 48 h with normal FBS (left) and CS–FBS medium (right).. (B) Intracellular ROS level in 22Rv1 cells by DCFH-DA assay (480 nm/530 nm). O.D. optical density. (C) The expression of AR-FL and AR-V7 expression in human prostate cancer cell lines, LNCaP, 22Rv1, and VCaP. The mRNA levels were measured by qRT–PCR. The primers used are listed in Table S1. GAPDH was used as an internal control. Protein levels were assessed by Western blotting analyses and AR antibody detected both AR-FL (110 kDa) and AR-Vs (80 kDa). β-actin was used as an internal loading control. (D) The mRNA levels of AR-FL and AR-Vs in 22Rv1 cells treated with luteolin for 48 h were analyzed by qRT–PCR. (E) Western blotting analyses for AR-FL, AR-V7, cl-caspases 3 and 7, caspases 3 and 7, Gpx2, and β-actin in 22Rv1 cells treated with luteolin for 48 h. (F) The proliferation rate in VCaP cells treated with luteolin for 48 h. Western blotting analyses for AR-FL, AR-V7, cl-caspase 7, caspase 7 and β-actin in VCaP cells treated with luteolin for 48 h. (G) Western blotting analyses for AR-FL, AR-V7, cl-caspases 3 and 7, caspases 3 and 7, and β-actin in 22Rv1 cells in CS–FBS treated with luteolin for 48 h. (H) The 22Rv1 cell line was transfected with either negative control (NC) siRNA, AR-FL siRNA or AR-V7 siRNA. Western blotting analyses for AR-FL and AR-V7. Each proliferation rate.

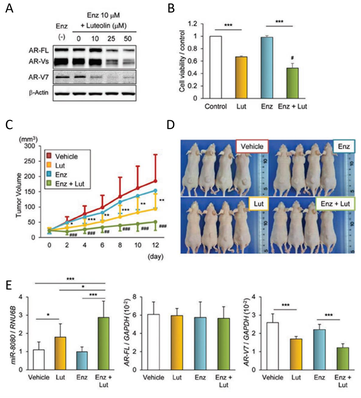


(III) Therapeutic effect of luteolin on CRPC tumor growth by suppression of AR-V7 in a 22Rv1 xenograft model.

(A) Tumor volume of 22Rv1 in castrated nude mice. Mice received a control diet (Ctrl) or a diet with luteolin (Lut; 100 ppm). The representative tumors in each group at 4 weeks after treatment. (B) Western blotting analyses for AR-FL, AR-V7 and β-actin in 22Rv1 xenografts. (C–E) The labeling indices for AR-V7 (C), TUNEL (D) and vessel density by CD31 immunohistochemistry (E) in 22Rv1 xenografts. Data are presented as mean ± SD, n = 20, $^{***}P <$ 0.001 statistically significant compared with control group.

(IV) Luteolin inhibits AR-V7 by induction of miR-8080 in 22Rv1 cells.

(A) Cells (22Rv1) were treated with 25 mM luteolin and/or 5 mM MG132 for 16 h. Western blotting analysis for AR-FL and AR-V7 was carried out. (B) *In silico* analysis using the miRbase Sequence Database to detect miRNAs that directly interact with AR-V7. The hsa-miR-8080 can bind the 3′-untranslated region of AR-V7. (C) Quantification of miR-8080 expression with luteolin (Lut), apigenin (Api) for 48 h or quercetin (Que) in 22Rv1 cells by qRT–PCR. Data are presented as mean ± SD, n = 4 per group, $^*P <$ 0.05 statistically significant compared with control group. (D) Western blotting analysis for AR-FL and AR-V7 with treatment of Lut, Api or Que for 48 h. (E, F) Effect of miR-8080 transfection on AR-V7 expression and cell proliferation in 22Rv1 cells. (E) The levels of AR-FL, AR-V7, cl-caspases 3 and 7, and caspases 3 and 7 were detected by western blotting. (F) Cell viability. (G, H) Effect of miR-8080 inhibitor on AR-V7 expression and cell proliferation in 22Rv1 cells, with or without luteolin. (G) Levels of AR-FL and AR-V7 were detected by western blotting. (H) Cell viability is presented as mean ± SD, n = 4 per group, $^*P <$ 0.05, $^{***}P <$ 0.001 statistically significant between the groups shown.



(V) MiR-8080 enhances the chemotherapeutic effect of enzalutamide in 22Rv1 cells both *in vitro* and *ex vivo*.

(A, B) Cells (22Rv1) were treated with enzalutamide (Enz), with or without luteolin (Lut) for 48 h. (A) Western blotting analyses for AR-FL and AR-V7. (B) Cell viability is presented as mean ± SD, n = 4 per group, #*P* < 0.05 statistically significant between Lut and Enz + Lut, and $^{***}P <$ 0.001 statistically significant between the groups shown. (C–F) Effect of luteolin on the chemotherapeutic efficacy of enzalutamide in 22Rv1 xenografts (1.0×10^6 cells) in castrated nude mice. Sixty mice were randomly divided into four groups: vehicle, Lut (luteolin 100 ppm in diet), Enz (enzalutamide 10 mg/kg/day, intraperitoneal injection, 5 times weekly), or Enz + Lut (luteolin 100 ppm + enzalutamide 10 mg/kg/day). (C) Tumor volumes of 22Rv1 xenografts are presented as mean ± SD in each week. n = 15 per group, $^*P <$ 0.05, $^{**}P <$ 0.01, $^{***}P <$ 0.001 statistically significant between Lut and vehicle groups, ###*P* < 0.01, ####*P* < 0.001 statistically significant between Lut and Enz + Lut group. (D) Gross morphology of subcutaneous 22Rv1 tumors at 2 weeks. (E) Quantitative gene expression of miR-8080, AR-FL, and AR-V7 by qRT–PCR.