Ursolic Acid Inhibits the Initiation, Progression of Prostate Cancer and Prolongs the Survival of TRAMP Mice by Modulating Pro-Inflammatory Pathways

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Abstract

Prostate cancer is one of the leading causes of cancer death among men worldwide. In this study, using transgenic adenocarcinoma of mouse prostate (TRAMP) mice, the effect of diet enriched with 1% w/w ursolic acid (UA) was investigated to evaluate the stage specific chemopreventive activity against prostate cancer. We found that TRAMP mice fed with UA diet for 8 weeks (weeks 4 to 12) delayed formation of prostate intraepithelial neoplasia (PIN). Similarly, mice fed with UA diet for 6 weeks (weeks 12 to 18) inhibited progression of PIN to adenocarcinoma as determined by hematoxylin and eosin staining. Finally, TRAMP mice fed with UA diet for 12 weeks (weeks 24 to 36) demonstrated markedly reduced tumor growth without any significant effects on total body weight and prolonged overall survival. With respect to the molecular mechanism, we found that UA down-regulated activation of various pro-inflammatory mediators including, NF-κB, STAT3, AKT and IKKβ phosphorylation in the dorsolateral prostate (DLP) tissues that correlated with the reduction in serum levels of TNF-α and IL-6. In addition, UA significantly down-regulated the expression levels of cyclin D1 and COX-2 but up-regulated the levels of caspase-3 as revealed by immunohistochemical analysis of tumor tissue sections. Finally, UA was detected in serum samples obtained from various mice groups fed with enriched diet in nanogram quantity indicating that it is well absorbed in the GI tract. Overall, our findings provide strong evidence that UA can be an excellent agent for both the prevention and treatment of prostate cancer.

Citation: Shanmugam MK, Ong TH, Kumar AP, Lun CK, Ho PC, et al. (2012) Ursolic Acid Inhibits the Initiation, Progression of Prostate Cancer and Prolongs the Survival of TRAMP Mice by Modulating Pro-Inflammatory Pathways. PLoS ONE 7(3): e32476. doi:10.1371/journal.pone.0032476

Editor: Ganesh Chandra Jagetia, Mizoram University, India

Received July 8, 2011; Accepted January 28, 2012; Published March 12, 2012

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Funding: This work was supported by grants from National Medical Research Council of Singapore [R-184-000-168-275 and R-184-000-201-275] to GS. APK was supported by grants from the National Medical Research Council of Singapore [Grant R-713-000-119-275] and Cancer Science Institute of Singapore, Experimental Therapeutics I Program [Grant R-713-001-011-271]. KMH was supported by a grant from the National Medical Research Council of Singapore, Biomedical Research Council of Singapore, and the Singapore Millennium Foundation. The funders has no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Prostate cancer is the second leading cause of cancer-related death among men in Western countries after lung cancer [1]. Chronic inflammation is increasingly being recognized as a mediator for many cancers [2,3,4] and considerable evidence suggests that it plays a major role both in the development and progression of prostate cancer [5]. First, patients with symptomatic prostatitis are more susceptible to developing prostate cancer. Second, prostate cancer has been associated with sexually transmitted infections. Third, decreased risk of prostate cancer is linked with increased intake of fruits and vegetables, antioxidants, and non-steroidal anti-inflammatory drugs (NSAID) [6,7]. Fourth, normal prostate undergoes proliferative inflammatory atrophy (PIA) before forming prostate intraepithelial neoplasia (PIN), the precursor of prostate cancer [8]. Fifth, PIA can overexpress inflammatory enzyme COX-2 [9]. Sixth, the transcription factors NF-κB and STAT3, both major mediator of inflammation, are constitutively active in prostate cancer tissues [10]. And seventh, NF-κB-regulated inflammatory cytokines such as interleukin (IL)-6 is an autocrine growth factor known to be secreted by prostate cancer tissues [11,12]. Therefore, it is reasonable to suggest that agents that can suppress inflammatory mediators have a potential for both the prevention and treatment of prostate cancer.

UA (3β-hydroxy-urs-12-en-28-oic acid), a pentacyclic triterpenoid derived from berries, leaves, flowers, and fruits of medicinal plants, such as Rosmarinus officinalis, Eriobotrya japonica, Calluna vulgaris, Ocimum sanctum, and Eugenia jambolana is one such agent that has been extensively studied for its anti-inflammatory and anticancer activities in the past decade [13]. UA has been reported to suppress the proliferation of a variety of tumor cells, to induce apoptosis, and to inhibit tumor promotion, metastasis, and angiogenesis [14,15,16,17]. Our group is currently investigating the unexplored potential of UA for the prevention and treatment of prostate cancer and has recently reported in two separate studies that UA can indeed suppress the growth of prostate
UA suppresses prostatic intraepithelial neoplasia (PIN) formation

It has been reported previously that TRAMP mice exhibit increased epithelial stratification in the dorsolateral prostate, epithelial cells with variably elongated nuclei with condensed chromatin with flat patterns of low-grade (LG) PIN (LG-PIN) by 4–8 weeks of age and tufted micropapillary and a cribriform pattern of HG-PIN by 6–10 week of age [22]. To investigate if UA suppresses PIN formation, 4-week-old mice were fed with UA (1%, w/w) containing diet for 8 weeks and sacrificed at 12 weeks of age (Group 1). The wet weight of the prostate with seminal vesicles did not appreciably change in both the control and UA fed TRAMP mice (data not shown). Visual examination of the abdominal cavity also did not reveal unusual enlargement of the seminal vesicles, prostatic lobes or pelvic lymph nodes. Consumption of UA enriched diet was well tolerated without evidence of toxicity in terms of animal appearance, behavior and body weight.

While non-TRAMP mice did not show any PIN formation (Fig. 1B, i), TRAMP mice animals showed LG- and HG-PIN and well differentiated carcinoma (WDC) (Fig. 1B, ii and iii). The effects of UA-treatment on the formation of PIN are shown in Fig. 1C. LG-PIN was seen in 60% of UA-treated mice compared to 40% in controls while HG-PIN was seen in 20% of UA-treated mice as opposed to 80% in controls. This demonstrated that UA treatment had reduced the formation of PIN as well as delayed the progression from LG- to HG-PIN. In addition, only one animal showed moderately (MDC) or poorly differentiated carcinoma (PDC) at this stage. These data strongly suggest that UA has the potential to be used as a chemopreventive agent.

UA prevents the progression from PIN to prostate cancer

To determine whether or not UA can prevent progression from PIN to prostate cancer, 12-weeks-old TRAMP-mice were fed with control or UA (1%, w/w) enriched diet for 6 weeks and then sacrificed at 18 weeks (Fig. 1A, Group 2). At 18 weeks of age, the DLP of TRAMP mice exhibited higher incidence of LG-PIN, HG-PIN, WDC and MDC (Fig. 2A, i & ii) when compared to those observed at younger ages (Fig. 1B & C). No significant changes in appearance, body weight and food consumption were observed between Group 2 mice and their controls. However, there was an appreciable but statistically insignificant decrease in the wet weight of prostate gland with the seminal vesicles in UA fed mice (data not shown). UA-treatment reduced the incidence of LG-PIN by 60%, HG-PIN by 40% and WDC by 40% (Fig. 2A, iii). Overall, there was a noticeable shift towards the higher incidences of non-cancerous DLP in UA-treated mice.

UA inhibits serum TNF-α and IL-6 levels in TRAMP mice

TNF-α and IL-6 are pro-inflammatory cytokines, and are considered as major biomarkers of inflammation. Groups 1, 2 and 3 mice together with their controls were sacrificed at the end of each treatment protocol; blood was collected via cardiac puncture. The serum obtained was evaluated for the levels of TNF-α and IL-6.

UA inhibits the activation of AKT, IKKα/β, NF-κB and STAT3 in DLP of TRAMP mice

We have recently reported that UA can inhibit AKT, IKKα/β, NF-κB and STAT3 activation in androgen dependent and independent prostate cancer cell lines [18]. In this study, we further examined the effects of UA enriched diet on the activation of pro-inflammatory mediators in the DLP of TRAMP mice (Fig. 1A). As shown in Fig. 2C, progressive increase in the phospho (p) -AKT, -pIKKα/β, pSTAT3, and p65 expression were observed from Group 1 to Group 3 TRAMP mice as the cancer gradually progressed from LG-PIN formation in group 1 at 4–12 week of age, HG-PIN and WD carcinoma in DLP in group 2 at 12–18 week of age, and WDC, MDC and PDC when compared to those observed at younger ages (Fig. 1B & C). No significant differences in age-matched non-TRAMP C57BL/6 mice, used as negative controls, did not show any change in the levels of these proinflammatory proteins. Interestingly, we observed that UA treatment resulted in approximately 40–50% inhibition in the expression of these phosphorylated proteins in the DLP of group 2 and group 3 as compared to untreated TRAMP mice, while only a slight decrease was observed in group 1 (Fig. 2C). The levels of the non-phosphorylated AKT and IKKα were minimally affected by UA-treatment (Fig. 2C). Taken together, our data clearly indicated that the suppression of pro-inflammatory AKT, NF-κB and STAT3 activation plays a role in the inhibition of the progression of prostate cancer in TRAMP mice by UA.

UA suppresses the growth of established prostate cancer in vivo

To determine if UA can suppress the growth of established prostate cancer, 24-week-old TRAMP-mice were fed with a control or UA (1%, w/w) enriched diet for 12 weeks and then sacrificed at the 36 weeks (Fig. 1A, Group 3). At this age, visual examination of the abdominal cavity clearly revealed enlarged seminal vesicles, DLP and other prostate lobes in the control TRAMP-mice. The DLP showed higher incidence of WDC, MDC and PDC when compared to those observed at younger ages, suggesting that a majority of the animals had cancerous lesions in the DLP (Fig. 2B, i & ii). In contrast, UA-treated TRAMP mice showed reduced incidence of HG-PIN by 40%, WDC by 60%, MDC by 40%, and PDC by 20% (Fig. 2B iii). Overall, the results indicated that UA-treated mice showed non-cancerous lesions ranging from LG-PIN and HG-PIN to normal stroma compared to 100% incidence of prostate cancer in control TRAMP mice.
6 using an ELISA kit. Both, TNF-α and IL-6 levels increased from Group 1 to Group 3 corresponding to increasing age and stages of cancer progression (Fig. 3A). UA-treatment significantly decreased serum TNF-α in Group 2 (by ~90%) Group 3 (~30%) (Fig. 3A, i) as well as serum IL-6 in all three groups by ~40–90% (Fig. 3A, ii).

UA suppresses the expression of cyclin D1, COX-2 and Caspase-3 in DLP of TRAMP mice

We next investigated various gene products involved in cancer progression (COX-2), proliferation (cyclin D1) and apoptosis (caspase-3) by immunohistochemistry in the DLP of Group 3.
TRAMP mice. As shown in Fig. 3B, UA-treatment reduced the number of cells stained positive for cyclin D1 from 31% to 3% and COX-2 from 89% to 27%. As expected, the expression of caspase-3 increased from 6% to 95%. These results clearly indicated that inhibition of multiple gene products involved in tumor progression and the induction of apoptosis contribute to the potent anti-tumor activities of UA as observed in TRAMP tumor tissues.

UA suppresses tumor growth and increases the survival of TRAMP mice
To determine if UA can suppress the growth of established prostate cancer, 24-week-old TRAMP-mice were fed with a control or UA (1%, w/w) enriched diet for 12 weeks and then sacrificed at the 36 weeks (Fig. 1A, Group 3). The wet weight of the prostate with seminal vesicles showed significant differences in
the weight of the whole prostate gland. A significant (P<0.05) difference in the tumor volume was observed in the UA-treated mice (Fig. 4A). We also found that the long term feeding of UA enriched diet is safe without any organ or tissue toxicity with minimal effect on body weight (Fig. 4B). Throughout the 36 weeks period, age matched non-transgenic C57BL/6 mice was used as a negative controls, which showed none of the features associated with prostate cancer observed in TRAMP mice. All data presented on the effects of UA-treatment in TRAMP mice (Groups 1 to 3) are consistent with the survival data indicating that such treatment can significantly prolong the life span of mice (Fig. 4C). In addition to Groups 1, 2 and 3, a 4th group of TRAMP-mice were fed with the UA enriched diet for 36 weeks from 4 weeks of age (Group 4); control groups received normal diet (Fig. 1A). Mice were scored at the time of death based on tumor burden. Kaplan-Meier survival plots were generated to determine the overall survival. Log-rank (Mantel-Cox) plots showed increases in the overall survival of mice in all UA-treated groups. Median survival of mice in the control group was 55 weeks, compared to 75 weeks for Group 1 and Group 2, and 72 weeks for Group 4 (P<0.05). In group 3, the median survival was 68 weeks but failed to reach statistical significance (Fig. 4C). Therefore, it may be concluded that UA-treatment is most effective when it was commenced early when the TRAMP-mice showed no cancer or only early stages of PIN development.

Detection of UA in serum

UA was detected in serum of all 3 groups of TRAMP-mice fed with UA enriched diet. The concentrations detected varied from about 600 to 1300 ng/ml with no statistical significance between groups (Fig. 5B). As UA serum levels were expected to have reached steady state concentration in all three groups, the differences in concentration thus reflected individual variations. No additional peaks were detected in the serum, indicating that UA does not generate any metabolites (Fig. 5A, iii and iv). Our study presents the first experimental evidence that UA is well absorbed after oral feeding and the serum concentrations attained are sufficient to elicit biological effects as evident by the inhibition of prostate tumor growth in TRAMP mice.
Discussion

The TRAMP mouse model is a well established spontaneously developing prostate cancer model that mimics natural prostate tumor progression, from PIN to highly invasive metastatic prostate cancer [20] in humans. In TRAMP mice, tumor originates from both DLP and ventral prostate (VP) as a result of Tag expression that is primarily restricted to the prostatic lobes [22], while human prostate cancer originates from the peripheral zone that is similar to the DLP in mice [23]. The anticancer effects of various chemopreventive agents, including oleanolic acid analogs, sulforaphane, polyphenols obtained from green tea, garlic constituents, curcumin, 3,5'-diindolylmethane and genistein have been evaluated previously on spontaneously developing prostate tumorigenesis in TRAMP mice [24,25,26]. In addition, lycopene, γ-tocopherol, silbinin and tomato enriched diet have all been found to increase survival and delay the progression from PIN to adenocarcinoma and decrease the incidence of poorly differentiated carcinoma [23,27,28,29,30]. However, chemoprevention study with UA in prostate cancer model has not been carried out previously, and only our group has recently reported for the first time that UA enriched diet can indeed inhibit prostate tumor metastasis by modulating the CXCR4/CXCL12 axis in TRAMP mice [19]; although the effect of UA on tumor development and progression was not evaluated. In the present study, we studied the effect of UA treatment at three different stages of cancer development in TRAMP mice. We found that UA can significantly inhibit cancer development and progression at all three stages. In Group 1 mice, we observed measurable suppressive effects of UA on the PIN development, while in Group 2 mice, development of high grade PIN was inhibited as
UA Modulates Tumor Growth in TRAMP Mice

Animal experiments were conducted in accordance with Singapore NACLAR guidelines (Law as of November 2004) for laboratory animal use and care and approved by NUS Institutional Animal care and Use Committee protocol number 052/09. Briefly, 4-week-old TRAMP female mice purchased from The Jackson Laboratory were mated with C57/BL/6 male at NUS CARE (Singapore). The pups were genotyped for the transgene (Tag) at 3 weeks of age; DNA was extracted from the tail snips using TRIZOL® reagent (Invitrogen, USA) and quantified using Nanodrop spectrophotometer (Thermo scientific, USA). One µg of the DNA was taken for PCR analysis. Polymerase chain reaction (PCR) reaction mixture contained 10 µl of 5x Qiagen PCR buffer, 1 µg of DNA, 0.6 µM each of forward and reverse primers, 2 µl of dNTP mix and 1 µl of Taq polymerase enzyme in a final volume of 50 µl. Primers used for genotyping, forward primer: 5’-CCG GTC GAC CGG AAG CTT CGA CAA GTG CAT TTA-3’, Reverse primer: 5’-AGG CAT TCC ACC ACT GCT CCC ATT CAT C-3’. PCR conditions were 94°C for 5 min, 94°C for 30 sec, 62°C for 1 min, 72°C for 1 min, repeat steps 2-4 for 35 cycles, 72°C for 2 min and held at 4°C. PCR products were run on 1% agarose gel containing 1X GelRed nucleic acid gel stain from Biotium (Hayward, CA). Stained bands products were run on 1% agarose gel containing 1X GelRed nucleic acid gel stain from Biotium (Hayward, CA). Stained bands were visualized under UV light and photographed using Biorad Gel doc EZ system (Bio-Rad, USA). Transgene positive pups were then used for the subsequent experiments.

Pathological grading of tumor tissues

The pathological grading of TRAMP prostate cancer was performed according to the grading system described previously [22]. Prostate lesions in the DLP were histologically graded as normal (duct lined with single layer of secretory epithelial cells surrounded by two or three cell layers of fibromuscular stroma), low-grade prostatic intraepithelial neoplasia (PIN) [epithelial cells with variably elongated nuclei with condensed chromatin], high-grade PIN (epithelial stratification and tufting, presence of micropapillary and cribiform structures), well-differentiated carcinoma (WDG) (epithelial cells invading fibromuscular stroma), and moderately differentiated carcinoma (MDC) to poorly differentiated carcinoma (PDC) adenocarcinoma of the prostate (sheets of neoplastic cells with little or no glandular structures). 15 randomly selected microscopic fields on hematoxylin and eosin (H&E) stained sections of the DLP were scored for the incidence and pathological grade of the prostate cancer in control and UA fed TRAMP mice.

Materials and Methods

Reagents

UA (98% pure) was purchased from Guangxi Changzhou Natural Products Development Company Ltd (China). UA was mixed with normal mouse diet at 1% w/w and stored at 4°C. Tris, glycine, NaCl, SDS, glycyrrhetinic acid and β-actin antibody were purchased from Sigma-Aldrich (St. Louis, MO, USA) and HRP-conjugated secondary antibody was obtained from Invitrogen (Carlsbad, CA, USA). Antibodies against phospho-STAT3 (Tyr 705), phospho-AKT [Ser 473], STAT3 were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Antibodies to AKT, p65, phospho-specific IKKα/β (Ser 180/181), and IKKα/β were purchased from Cell Signaling Technology (Beverly, MA). TNF-α and IL-6 ELISA kits were purchased from Research Instruments (MA, USA). Bradford reagent was purchased from Bio-Rad (Hercules, CA). Immunohistochemistry staining kit was purchased from Dako (Denmark).

In vivo anti-tumor study

The inbred male TRAMP mice, 4 weeks old, were maintained in temperature controlled rooms with a 12 h light/dark cycle. All mice were weighed before start of experiment. The mice were then randomized into the following UA fed and control groups (n = 5). Group 1: 4 weeks old TRAMP mice were fed with UA (1% w/w) enriched diet for 8 weeks; Group 2: 12 weeks old TRAMP mice were fed with UA enriched diet for 6 weeks; Group 3: 24 weeks old TRAMP mice were fed with UA enriched diet for 12 weeks. Control TRAMP mice received normal diet. Body weight and tumor size were recorded twice every week and the tumor size was determined by Vernier caliper and calculated using the formula [length×width×height]/2. Mice were euthanized by CO2 inhalation followed by cervical dislocation. Blood samples, collected by cardiac puncture, were kept at 4°C overnight and centrifuged at 10,000 rpm for 20 min to obtain serum, which was stored in aliquots at −80°C. Tumor volume was measured and weighed. Prostate gland was microdisected from the seminal vesicles under a
stereomicroscope, fixed in 10% phosphate buffered formalin for H&E and immunohistochemical analysis.

ELISA assay for TNF-α and IL-6

Stored serum samples were analyzed for TNF-α and IL-6 levels using ELISA kit (R&D systems, USA) according to manufacturer instructions. Sandwich ELISA protocols were used and calibration was done with standard TNF-α and IL-6 provided in the kit.

Western blotting

Dorsolateral prostate tumor tissues were lysed in lysis buffer containing (20 mM Tris (pH 7.4), 250 mM NaCl, 2 mM EDTA (pH 8.0), 0.1% Triton X-100, 0.01 mg/ml aprotinin, 0.005 mg/ml leupeptin, 1 mM PMSF, and 4 mM NaVO4). Lysates were then centrifuged at 13,000 rpm for 10 min to remove insoluble material and 50 μg of protein was resolved on a 10% SDS gel. After electrophoresis, proteins were electrotransferred to a nitrocellulose membrane, blocked with 5% nonfat milk, and probed with antibodies of interest overnight at 4°C. The blot was washed, exposed to HRP-conjugated secondary antibodies for 1 h, and finally examined by chemiluminescence (ECL; GE Healthcare, Little Chalfont, Buckinghamshire, UK). Densitometric analysis of the scanned blots was performed using Image J software and the results are expressed as fold change relative to control.

Immunohistochemistry

DLP tumor tissues were fixed with 10% phosphate buffered formalin, processed and embedded in paraffin. Tissue sections, 5 μm, were cut and deparaffinized as described previously [18]. Images were taken using a Olympus BX51 microscope (magnification, 20×). Positive cells (brown) were quantitated using The Image-Pro plus v6.3 software package (Media Cybernetics, Inc.).

Survival studies

To investigate whether UA enriched diet can prolong the life span of TRAMP mice, 25 male inbred TRAMP male mice were divided into control and UA fed groups (n = 5). Group 1: 4 weeks old TRAMP mice were fed with UA enriched diet for 8 weeks; Group 2: 12 weeks old TRAMP mice were fed with UA enriched diet for 6 weeks; Group 3: 24 weeks old TRAMP mice were fed with UA enriched diet for 12 weeks; Group 4: 4 week old mice were fed with UA enriched diet for 36 weeks. Control TRAMP mice received normal diet. Animals were monitored weekly for body weight and tumor development by abdominal pelvic palpation and survival.

Determination of UA in serum samples

Extraction of UA from mouse serum samples were carried out by liquid-liquid extraction (LLE) as previously described [32]. UA concentrations in mice serum samples were then determined using ultra performance liquid chromatography-mass spectrometry (UPLC-MS) as previously described with modifications [33]. Mass spectrometry data acquisition and subsequent analyses were carried out using linear ion trap quadrupole mass spectrometer (3200 QTRAP) equipped with a TurboIonSpray electrospray ionization (ESI) source and Analyst 1.4.2 software (Applied Biosystems, Foster City, CA, USA). Quantitation of analyte and internal standard (IS) was done using single ion monitoring in multiple reactions monitoring mode (MRM, m/z 455.0 and m/z 469.0 were used for UA and IS, respectively) [18]. Representative chromatograms are shown in Fig. 5A. The serum concentration of UA was calculated using Analyst software 1.4.2 (Applied Biosystem, USA).

Statistical analysis

Experiments were carried out in triplicates and repeated twice. The significance of differences between groups was evaluated by Student’s t-test and a p value of less than 0.05 was considered statistically significant. Kaplan-Meier survival plots were generated to determine the survival of mice and log-rank (Mantel-Cox) test was performed to determine the significance difference between control and UA fed groups.

Author Contributions

Conceived and designed the experiments: GS MKS KMH. Performed the experiments: MKS THO C.KL. Analyzed the data: MKS GS KMH PCH. Contributed reagents/materials/analysis tools: GS APK THO C.KL. Wrote the paper: MKS PTHW GS. Critically analyzed the manuscript: MKS PTHW GS. Conceived and designed the experiments: GS MKS KMH. Performed the experiments: MKS THO C.KL. Analyzed the data: MKS GS KMH PCH PTHW. Contributed reagents/materials/analysis tools: GS APK THO C.KL. Wrote the paper: MKS PTHW GS. Critically analyzed the manuscript: MKS PTHW GS.

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