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KAVA chalcone, Flavokawain A, inhibits urothelial tumorigenesis in the UPII-SV40T transgenic mouse model

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Abstract

Flavokawain A (FKA) is the predominant chalcone identified from the kava plant. We have previously demonstrated that FKA preferentially inhibits the growth of p53 defective bladder cancer cell lines. Here we examined whether FKA could inhibit bladder cancer development and progression *in vivo* in the UPII-SV40T transgenic model that resembles human urothelial cell carcinoma (UCC) with defects in the p53 and the retinoblastoma (RB) protein pathways. Genotyped UPII-SV40T mice were fed orally with vehicle control (AIN-93M) or FKA (6 g/kg food; 0.6%) for 318 days starting at 28 days of age. More than 64% of the male mice fed with FKA-containing food survived beyond 318 days of age, whereas only about 38% of the male mice fed with vehicle control food survived to that age (p= 0.0383). The mean bladder weights of surviving male transgenic mice with the control diet *versus* the FKA diet were 234.6 ± 72.5 *versus* 96.1±69.4 mg (P=0.0002). FKA was excreted primarily through the urinary tract and concentrated in the urine up to 8.4 μmol/L, averaging about 38 times (males) and 15 times (females) more concentrated than in the plasma (P=0.0001). FKA treatment inhibited the occurrence of high-grade papillary UCC, a precursor to invasive urothelial cancer, by 42.1%. A decreased expression of Ki67, survivin and XIAP and increased expression of p27 and DR5 and number of TUNEL-positive apoptotic cells were observed in the urothelial tissue of FKA-fed mice. These results

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Acquisition of data: Z. Liu, X. Xu

Analysis and interpretation of data: Z. Liu, X. Zi

Administrative, technical, or material support: X. Zi, X. R. Wu

Writing, review, and/or revision of the manuscript: X. Zi, Z. Liu, X. R. Wu, A. R. Simoneau

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suggest a potential of FKA in preventing the recurrence and progression of non-muscle invasive UCC.

Keywords

Kava; bladder cancer; UPII-SV40T transgenic mice

Background

Bladder cancer is the fourth most common cancer in men and eighth most common in women in the United States [1]. Bladder cancer has been categorized into non-muscle-invasive bladder cancer [NMIBC, pTa, pT1, and carcinoma *in situ* (CIS)] and muscle-invasive bladder cancer (MIBC, pT2-4), depending on whether or not tumor infiltration extends into the muscularis propria of the bladder wall [2–4]. NMIBC is treated mainly by transurethral resection with or without intravesical therapy [5]. Tumors often recur and some progress to invasive or metastatic urothelial cell carcinoma (UCC). Muscle-invasive UCCs require radical cystectomy or intravenous chemotherapy with radiation protocols [6]. Treatment options for metastatic bladder cancers are extremely limited, with 6 % five-year survival rate and median survival time of 12 to 20 months [6]. Therefore, there is a great need to develop improved treatment for bladder cancer. Because the high recurrence rate of NMIBC requires repeated cystoscopy and resection is onerous to the patient and costly to the healthcare system, and development of muscle invasive or metastatic disease is debilitating or fatal, efforts focused on secondary prevention --preventing recurrences and progression to invasive and metastatic bladder cancer in those with papillary UCC and CIS -- should be a priority.

Studies from whole-bladder histological maps of human cystectomy specimens suggest that bladder cancer arises via two distinct but somewhat overlapping molecular pathways [7, 8]. Loss of chromosome 9 sequences has been considered an early event for both NMIBC and MIBC [8. 9]. Activation of the receptor tyrosine kinases (RTK)-Ras pathway through mutations in the H-Ras and FGFR-3 genes, as well as overexpression of H-Ras, FGFRs, and ERBB3 and 4 have been frequently found in 70–90% of NMIBC [7, 10], whereas inactivation of p53 and pRB tumor suppressors (more than 50%) is believed to initiate a progressive genetic instability and accumulation of genetic defects, leading to MIBC [8, 9], Since bladder cancer is complex and heterogeneous, its risk stratification with different genetic and molecular alterations and development of targeted agents would allow more effective management of this disease.

FKA is a novel chalcone isolated from the Kava plant. Chalcones are α, β-unsaturated ketones and are unique in the flavonoid family [11]. They are the intermediate precursors for all flavonoids in the phenylpropanoid pathway in plants [11]. Given that both citrus fruits, apples and other plant-derived dietary products are rich dietary sources of chalcones [12–15], daily intake of chalcones by people could be significant. Flavonoids, including chalcones, and their metabolites are excreted from the kidney and concentrated in the urine [16], making flavonoids highly attractive agents in bladder cancer prevention. An *in vitro* studies have shown that FKA preferably inhibited the growth of different types of cancer cell lines (RT4, T24, UMUC3, TCCSUP, 5637, HT1376, and HT1197) with minimal effect on the growth of normal cells from different organs (breast, liver, prostate, skin, intestine, and bone marrow) and liver cell lines (i.e. L-02 and HepG2) at concentrations of up to 100 μM [17–20 and data not shown]. We have demonstrated that FKA was a potent inducer of apoptosis in bladder cancer cell lines via activation of death receptor 5 (DR5) and mitochondria-mediated apoptosis pathways and down-regulation of the expression of anti-

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apoptotic proteins: Survivin and XIAP [20, 21]. In addition, FKA exhibited greater growth inhibition of bladder cancer cell lines with mutant p53 than those with wild-type p53 [22]. FKA also exhibited *in vivo* anti-tumor activity in a bladder cancer xenograft model [21]. These results suggested that FKA deserve further investigation as a novel agent for targeting bladder cancer with p53 deficiency.

Urothelium-specific expression of SV40 large T antigen (SV40T), which is driven by the urothelium-specific uroplakin II (UPKII) promoter, results in the allelic loss of the p53 gene and functional inactivation of p53/pRb proteins in the mouse bladder [23, 24]. The UPII-SV40T transgenic mice develop bladder CIS, high-grade superficial papillary tumors (precursors to recurrent or invasive UCC), and high-grade UCC co-existing with muscle invasive UCC and/or metastasis. The rate of muscle-invasion or metastasis is about 17% [24]. Death is commonly from urinary obstruction and renal failure [24]. The number of transgene copies influences the rate of progression. High copies of UPII-SV40T mice rapidly progress to death in 3–6 months. Mice with low copies will progress to high grade disease more slowly, by 10 months allowing more time for intervention. Therefore, this model was used for testing the potential of FKA for preventing bladder cancer recurrence and progression. We found that FKA significantly reduces the tumor burden in this model and extends the survival of bladder cancer bearing mice, as well as decreases the occurrences of high-grade, papillary UCC. The *in vivo* mechanisms of FKA's action are associated with anti-proliferation and induction of apoptosis via down-regulation of XIAP and survivin and up-regulation of DR5 and p27.

Materials and Methods

Mouse breeding, Southern blotting and genotyping

Low copy male and female transgenic mice were selected for the experimental protocol. Wild type or nontransgenic were utilized in safety studies or as controls. Transgenic mice harboring high copy numbers (i.e. 6–10) of UPII-SV40T transgenes were excluded in the trial because of rapid progression with deaths in 3–6 months. Heterozygous UPII-SV40T+/− (FVB/N) females were cross-bred with heterozygous UPII-SV40+/− males. Genomic DNA was isolated from tail biopsies of individual transgenic mice using a protease K digestion and NaCl precipitation method as described by Cheng et al. [24]. DNA was digested with NcoI, resolved by gel electrophoresis, and hybridized with a 550-bp BamHI-StuI probe located at the 3′ end of the mouse UPII promoter to identify a transgene fragment (6.3 kb) and an endogenous UPII gene fragment (1.4 kb) as described by Cheng et al. [24]. The transgene dosage was determined by comparing the density of a transgene band to that of the endogenous UPII gene band on scanned-films. Heterozygous mice have an approximately 1:2 ratio of the transgene to the endogenous UPII gene, whereas the homozygotes have the 1:1 ratio of the transgene to the endogenous UPII gene.

Experimental animal groups, treatment and necropsy

Four-week-old, genotyped low-copy UPII-SV40T transgenic mice were fed with diet supplemented with vehicle control or that with 0.6% FKA [0.6% FKA (w/w) in AIN-93M purified] and then sacrificed at ages of 90 or 318 days (Figure 1A). In parallel, age-matched nontransgenic mice (n = 6 mice per group) were fed control or 0.6% FKA diet as overall controls for the same durations. A randomization process was used to ensure a comparable initial body weight in each group. All diets were prepared commercially (Dyets, Inc.). Mice were permitted free access to food and water. All animals were examined daily for morbidity, mortality, clinical signs of ataxia, and toxicological effects including respiratory depression, neurobehavioral abnormalities, color of skin and eyes (a sign of liver toxicity), and motor activity. Food consumption and animal body weight were recorded biweekly.

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Animal care and treatments were in accordance with Institutional guidelines and the approved protocol by UCI (protocol #:2004-2540).

At the end of treatment, all mice were euthanized by CO2 asphyxiation. Serum samples were obtained by cardiac puncture. Urine samples were obtained by bladder massage. A laparotomy was performed to expose all major organs which were inspected for frank toxicity and any visible abnormality. Photographs were taken by a digital camera to document the animals and their urogenital systems *in situ*. All non-bladder organs were removed and fixed in formalin for standard H&E slide preparation and examination. Any evidence of edema, abnormal organ size, or appearance in non-bladder organs was noted. A portion of the urinary bladder was fixed in 10% neutral-buffered formalin for histopathological evaluation and the rest was snap frozen in liquid nitrogen and stored at −80°C for further analysis. Sections of each urinary bladder tumor were histologically evaluated by a pathologist blinded to the experimental groups. Histological lesions were classified into dysplasia (urothelium with increased cell size, nuclear pleomorphism, and hyperchromatism), *CIS* (thickened urothelia with profound nuclear atypia, crowding, high nuclear/cytoplasmic ratio, frequent mitotic figures, and loss of cellular polarity), high-grade papillary UCC (a poorly differentiated urothelial cell carcinoma), and high-grade papillary UCC co-existing muscle-invasive UCC (urothelial cell carcinoma invades into the muscular layer of the bladder) or metastasis.

Measurement of FKA concentrations in plasma and urine by Ultra Performance Liquid Chromatography (UPLC)/Tandem Mass Spectrometry (MS/MS)

FKA was extracted from plasma and urine samples by acetonitrile, and then filtered with 0.45 μm solvent resistant filters and stored at −80°C until further analysis. Chromatography was performed on an Acquity UPLC system (Waters Corp., Milford, MA, USA) with an auto-sampler at 8°C. Separation of compounds was carried out at 50°C using a Waters Acquity UPLC ®BEH C18 1.7 μm (2.1×50 mm) column with a gradient elution: (A) Water: Acetonitrile: acetic acid (97.8:2:0.2 v/v/v), and (B) Acetonitrile: acetic acid 99.8:0.2 (v/v) as the mobile phase. The elution program was as follows: 10% B (initial), 90% B (1.0 min), 90% B (2.0 min), 10%B (2.05 min), and 10% (3 min). The flow rate was 0.3 mL/min and the injection volume was 10 μL. The UPLC was coupled to Micromass Quattro Micro Liquid chromatography–mass spectrometry (LC/MS/MS) triple quadrupole mass spectrometer (Mass Range: 2~2000 m/z) with electrospray ionization (ESI) interface in positive mode. The instrument was operated in a positive ion mode with an ESI voltage of 3.8 kV, and a desolvation gas flow of 700 L/h. Argon was used as the collision induced dissociation gas at a pressure of 7.1e–3 mbar with a selected reaction monitoring transition for FKA of m/z 315>m/z 181 and flavokawain B (internal control) of 284>181

Immunohistochemistry

Paraffin-embedded sections (5-μm thick) were heat immobilized, deparaffinized using xylene, and rehydrated in a graded series of ethanol with a final wash in distilled water. Antigen retrieval was done in 10 mM citrate buffer (pH 6.0) in a pressure cooker for 6 and 19 min at 125 °C and 90 °C, respectively. The sections were then incubated with mouse monoclonal anti-Ki-67 antibody (abcam, 1:800), anti-Survivin (Cell Signaling, 1:200), anti-death receptor-5 (DR5) (abcam, 1:100) and anti-p27/Kip (BD, 1:100) for 1 h at 37°C in a humidity chamber. Negative controls were treated only with PBS under identical conditions. In addition, negative isotypic IgG controls were used for validating the specificity of these antibodies. The sections were then incubated with biotinylated rabbit anti-mouse IgG (1:200 in 10% normal goat serum) for 30 min at room temperature. The sections were then incubated with 3, 3′-diaminobenzidine (DAB) as described in R&D systems Cell & Tissue Staining Kit instructions. The sections were finally counterstained with diluted Harris

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hematoxylin (Sigma Chemical Co.) for 2 min, and rinsed in Scott's water. Proliferating cells were quantified by counting the Ki67-positive cells and the total number of cells at 12 arbitrarily selected fields at × 200 magnification in a double-blinded manner.

DeadEnd ColorimetricTUNEL Assay

Apoptotic cells were detected using the DeadEnd Colorimetric TUNEL system (Promega, WI) following manufacturer's protocol. Paraffin-embedded sections (5-μm thick) were heat immobilized, deparaffinized using xylene, and rehydrated in a graded series of ethanol with a final wash in distilled water. The sections were then treated with proteinase K (30 μg/ml) for 1 h at 37°C, followed by 3% hydrogen peroxide for 10 min. After thorough washing with PBS, sections were incubated with equilibration buffer for 10 min, and then TdT reaction mixture was added to the sections, except for the negative control, and incubated at 37°C for 1 h. The reaction was stopped by immersing the sections in 2X SSC for 15 minutes. After equilibrating and washing, sections were incubated with conjugated horseradish peroxidase streptavidin (1:500) for 30 min at room temperature. Then substrate DAB was added until color development. The sections were finally counterstained with diluted Harris hematoxylin (Sigma Chemical Co.) for 2 min, and rinsed in Scott's water. Apoptotic cells were quantified by counting the TUNEL-positive cells and the total number of cells at 12 arbitrarily selected fields at × 200 magnification in a double-blinded manner.

Western blotting

Urothelial cells and tumor tissues were scraped off from the urinary bladders of FKA or vehicle control-treated UPII-SV40 mice and were homogenized in lysis buffer [Tris 20 mM (pH 7.5) containing EDTA, EGTA, Triton X-100 and proteinase cocktail inhibitors] using a Polytron homogenizer. Protein concentration was determined by Biorad DC protein assay. Ten to 50 μg protein was resolved on 8 to 12% Tris–glycine gel, transferred onto nitrocellulose membranes and blocked for 1 h at room temperature with 5% non-fat dry milk/TBS solution. The membranes were then incubated with the required primary antibody (DR5, p27/Kip1, Bcl2, XIAP and survivin antibodies from Cell Signaling Technologies, Danvers, MA) overnight at 4°C and then with an appropriate secondary antibody. Protein was visualized by enhanced chemiluminescence detection system.

Statistical analysis

Prism statistic software was used to compute mean, standard deviations and confidence intervals of all quantitative data. Tumor, organ and body weight comparisons between vehicle control and FKA treatments were accomplished using either analysis of variance (ANOVA) or Student's t-test followed by the Bonferroni t-test for multiple comparisons. Survival analysis was performed using Log-rank test, and survival curves were computed by using the product limit method of Kaplan and Meier. Chi-square test was used to compare the percentages of mice with different pathological stages between vehicle control and FKA treatments. All statistical measures were two-sided, and P-values <0.05 were considered to be statistically significant.

Results

FKA feeding increases the survival of male low-copy UPII-SV40T transgenic mice

The UPII-SV40 transgenic line F19 harboring 2 copies of the transgene were shown to follow the sequential steps of tumor progression from dysplasia, carcinoma *in situ*, to high-grade papillary carcinoma [23]. Most of these mice are viable for more than six months and suitable for pre-clinical studies of the chemopreventive effects of novel agents for preventing cancer recurrence and progression in human urinary bladder cancer patients with

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carcinoma *in situ*. Therefore, a cohort of the UPII-SV40T transgenic mice were genotyped by Southern blotting and randomized into vehicle control diet (AIN-93M) or diet supplemented with 6 g/kg FKA (0.6%), and then sacrificed at 90 or 318 days of age (Figures 1 A and B). We aimed to determine whether dietary administration of FKA to low-copy UPII-SV40T transgenic mice during the full process of carcinogenesis increases the survival of the mice.

FKA feeding for about 290 days did not significantly affect average food consumption compared to vehicle diet feeding (Supplementary Figure 1). It appeared that FKA feeding slightly increased body weight, but it was not statistically significant (Figure 1C). About 38% of male UPII-SV40T transgenic mice fed with vehicle diet (n=22) survived over 318 days, whereas about 64% of 0.6% FKA fed male UPII-SV40T mice (n=22) survived more than 318 days of age. FKA feeding significantly increased the survival rate of male UPII-SV40T mice by absolute 26% (Figure 1D, Log-rank test, p=0.0383). About 89 and 91% of female UPII-SV40T mice fed with control and FKA, respectively, survived beyond 318 days (supplementary Figure 2). The comparison of the survival between control- and FKA-fed female UPII-SV40 mice therefore has not been extended beyond the 318 period due to prohibitively high cost of animal maintenance.

FKA feeding reduces weight of tumor bearing urinary bladders

Figure 2A shows that, between vehicle control- and FKA-fed wild-type male mice, there was no significant difference in the mean bladder weight, used as a surrogate for tumor growth at 90 days of age [Vehicle control *vs.* FKA; 14.9 ± 0.7mg (n=6) *vs.* 14.1± 2.8 mg (n=6), P>0.05] and at 318 days of age [Vehicle control *vs.* FKA; 21.0 ± 2.3mg (n=6) *vs.* 19.9 ± 3.4 mg (n=6), P>0.05], respectively. Due to tumor growth in the bladder, the mean bladder weights of male UPII-SV40T transgenic mice significantly increased by 2.5 fold at 90 days of age and 11.2 fold at 318 days of age, respectively, compared to those of age-matched wild-type litter mates (Ps<0.01; Figure 2A). FKA feeding significantly decreased the mean bladder weights of male UPII-SV40T transgenic mice by 27% [Vehicle control *vs.* FKA; 37.3 ± 12.8mg (n=24) *vs.* 27.1 ± 6 (n=21); P= 0.0017] at 90 days of age and by 59% [Vehicle control *vs.* FKA; 234.6 ± 72.5mg (n=9) *vs.* 96.1 ± 69.4 (n=14); P= 0.0002] at 318 days of age, respectively (Figure 2A). The inhibitory effect of FKA feeding on the bladder weights of male UPII-SV40T transgenic mice at 318 days of age could be underestimated in this study as mice bearing larger bladder tumors could not survive to 318 days because of outlet obstruction and renal failure.

In female wild-type mice, FKA feeding also did not significantly affect their mean bladder weights at 90 days of age [Vehicle control *vs.* FKA; 9.1± 1.3mg (n=5) *vs.* 10.1 ± 1.5 mg (n=6), P>0.05] or at 318 days of age [Vehicle control vs. FKA; 14.6± 5.2 mg (n=7) *vs.* 17.3± 4.9 mg (n=6), P>0.05] (Figure 2B). In female UPII-SV40T transgenic mice, the mean bladder weight increased by 2.3 fold by 90 days of age and 2.9 fold by 318 days of age, respectively, compared to those of age-matched wild-type litter mates (Ps<0.01; Figure 2B). Compared to male UPII-SV40T mice, female UPII-SV40T transgenic mice exhibited a significantly slower increase in bladder weight. FKA feeding also significantly reduced the mean bladder weights of female UPII-SV40T transgenic mice by 9% [Vehicle control *vs.* FKA; 21.2 ± 3.2 mg (n=24) *vs.* 19.2 ± 3.6 (n=26); P= 0.044] at 90 days of age and by 30% [Vehicle control vs. FKA; 42.3 ± 15.2 mg (n=25) *vs.* 29.4 ± 12.6 (n=24); P= 0.0023] at 318 days of age, respectively (Figure 2A).

At the end of the treatments, gross anatomy of wild-type mice revealed no evidence of edema, abnormal organ size, or changes in appearance in liver, spleen, colon, heart, kidney, lung, heart, seminal vesicle, and prostate. Figures 2C and D show that there was no

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difference in organ to body weight ratios in spleen, liver, lung, kidney, seminal vesicle and prostate between vehicle control- and FKA-fed wild type mice after treatment for 290 days.

FKA feeding reduces the occurrence of high-grade papillary tumors

Gross anatomy of the urogenital system in UPII-SV40T transgenic mice revealed bigger sizes of kidney, ureter and bladder, and the transgenic mice fed with FKA exhibited a smaller sized bladder (Figure 3A). Microscopic analysis of H&E-stained sections classified pathological features of urothelium into four classes: (a) dysplasia, (b) *carcinoma in situ*, (c) high-grade papillary carcinoma and (d) muscle-invasive UCC (Figure 3B). At the time of sacrifice in III and IV groups (the cohort of mice were followed up until 318 days of age or death), histopathologic evaluation showed that bladder tissues in vehicle control fed UPII-SV40T male mice developed either *carcinoma in situ* (22.2%, 2/9) or high-grade papillary carcinoma (66.7%, 6/9) and high-grade papillary carcinoma co-existing with muscle-invasive UCC (11.1%, 1/9). In FKA-fed SV40T male transgenic mice, only 35.7% (5/14) of the bladders exhibited high-grade papillary carcinoma and none of examined bladder tissues showed evidence of muscle-invasive UCC (Figure 3C, Chi square test, p=0.0487). About 30.2% of vehicle control fed UPII-SV40T female mice developed high-grade papillary UCC, whereas 16% of FKA fed female mice displayed high-grade papillary UCC (Figure 3D, Chi square test, p=0.1584). These results suggested that FKA feeding delayed the progression of *carcinoma in situ* to high-grade papillary and muscle-invasive UCC.

FKA concentrations in plasma and urine and their relationship with bladder weights in UPII-SV40T transgenic mice

We have established UPLC-MS/MS method for analysis of FKA in mouse plasma and urine (Figure 4A). Standard curves were linear with a regression coefficient greater than 0.9988 for calculation of FKA concentrations in mouse plasma and urine. FKA was undetectable in vehicle control-fed UPII-SV40T transgenic mice. FKA concentrations in plasma of FKA-fed UPII-SV40T transgenic mice ranged from 8.8 to180.4 ng/ml in males (n=10) and from 16.1 to 556 ng/ml in females (n=11), respectively (Figure 4B). The ranges of urine FKA concentrations in FKA-fed UPII-SV40T transgenic mice are from 208.6 to 2631.4 ng/ml in males (n=8) and from 377.2 to 2527.4 ng/ml in females (n=11) (Figure 4B). FKA concentrations in the urine were about 38 and 15 times higher than in the plasma in FKA-fed UPII-SV40T males and females, respectively. This result suggests that FKA may be metabolized and excreted into the urine at a faster rate in males than in females.

Figures 4C and D show that tumor bearing bladder weights of FKA-fed UPII-SV40T transgenic mice were inversely related to FKA concentrations in both plasma ($\gamma = -0.2728$) and urine ($\gamma = -0.4646$). It appears therefore that urine FKA concentrations may be more relevant to anti-bladder tumor effects of FKA.

FKA feeding decreases proliferation and increases apoptosis in bladder tissues

Microscopic examination of IHC stained bladder tissue sections showed a decreased number of Ki-67 positive cells in the FKA-treatment group compared to the control group. The percentage of Ki67 positive cells in the bladder tissues of FKA-fed male UPII-SV40 transgenic mice was 36.6 ± 4% compared with 21.6 ± 4.5 % in those of vehicle control-fed mice (P<0.01) (Figures 5A and B). This finding suggests an *in vivo* anti-proliferative effect of FKA on bladder tumor tissues thus slowing the progression of bladder cancer.

In contrast, Figure 5C show an increased number of TUNEL-positive apoptotic cells in the FKA-fed group. The percentage of TUNEL-positive cells in the bladder tissues of FKA– *vs.* vehicle control-fed UPII-SV40T transgenic mice was 37.8 ± 4.9% *vs.* 25.6 ± 4% (P < 0.01).

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This suggests a proapoptotic effect of FKA as another *in vivo* mechanism in the UPII-SV40T bladder tumor model.

FKA feeding affects the expression of apoptosis and cell cycle regulators

Immunohistochemical analysis shows that bladder tissue sections from FKA-fed male UPII-SV40T transgenic mice exhibited a significant increase in both the intensity and the number of DR5 and p27 positive cells and a decrease in survivin positive cells compared to those of vehicle control-fed mice (Figures 6A, B and C). Consistent with the *in vitro* results in bladder cancer cell lines reported previously [18–22], Western blotting analysis demonstrates that FKA feeding increased the protein levels of p27 and DR5, and down-regulated the levels of anti-apoptotic proteins: Bcl2, XIAP and survivin in bladder tissues of UPII-SV40T male transgenic mice (Figure 6D).

Discussion

About 70 to 80% of human urinary UCC bladder cancer patients present with NMIBC (pTa, pT1, and CIS) [3]. NMIBC (superficial tumors) can be effectively treated with bladder-sparing approaches using cystoscopy, trans-urethral resection of bladder tumor (TURB-T) and intravesical treatment (i.e., BCG) [25]. However, NMIBC has the highest recurrence rate of all cancers and requires lifelong, frequent and costly follow-up procedures (cystoscopy and biopsy) to test and treat for tumor recurrence and progression [25]. As a result, human urinary bladder cancer is the most expensive cancer to treat on a per-patient basis [26]. Therefore, our long-term goal is to develop low-cost and natural product-based agents for prevention and treatment of the recurrence and progression of NMIBC. In this study, we provided the first evidence that FKA, a naturally-occurring chalcone in the kava plant, effectively increases the survival of bladder tumor bearing mice and reduces tumor growth and the occurrence of high-grade papillary UCC in the UPII-SV40T transgenic model.

Transgenic mice harboring high copy numbers (i.e. 6–10) of UPII-SV40T transgenes develop invasive and metastatic bladder cancers, and often die at 3–5 months of age [23]. In this study, the low-copied (2 copies) UPII-SV40T transgenic mice were chosen. The low-copied UPII-SV40T transgenic mice progressively develop urothelium-specific dysplasia, CIS and high-grade papillary UCC due to defects in p53/Rb pathways [23, 24]. About 38% and 89% of male and female UPII-SV40T transgenic mice, respectively, survive beyond 318 days of age. About 22.2% of male UPII-SV40T transgenic mice exhibit CIS, and about 77.8% of these mice develop high-grade papillary UCC with or without muscle-invasion at 318 days of age. Compared to the high-copied UPII-SV40T transgenic mice and low-copied female transgenic mice, the low-copied male transgenic mice appear to follow more physiologically relevant steps of tumor progression within a reasonable experimental study period of time. In addition, high-grade papillary UCC has been considered to be a major precursor for invasive TCCs and is a major challenge in clinical management of human urinary bladder cancer [27, 28]. Therefore, the low-copied UPII-SV40T male transgenic mouse is a plausible model for evaluating the usefulness of chemopreventive agents in preventing and treating recurrence and progression of human urinary bladder with defective p53/Rb pathways. We demonstrated in this model that FKA treatment resulted in a reduction of high-grade papillary TCC with or without muscle-invasion by 42.1% and increased the survival of bladder tumor bearing mice by 26% at 318 days of age.

Dietary feeding of UPII-SV40T transgenic mice with 0.6% FKA shows that FKA was excreted through the urinary tract and concentrated in urine reaching about 8.4 μmol/L, whereas the maximum concentration of FKA in plasma was only about 1.8 μmol/L (Figure 4B). In addition, higher FKA concentrations in the urine appeared to be associated with a

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stronger anti-tumor growth effect of FKA in UPII-SV40T transgenic mice (Figures 4C and D). However, our study has limitations of not accurately measuring daily food consumption of each mouse and only detecting FKA concentrations in Plasma and urine at one time point. The amount of food consumption by individual mouse at different time points may affect their intake of FKA from the food, thereby contributing to the variation of FKA measurements in plasma and urine. FKA feeding also had a time-dependent anti-tumor growth effect. Treatment of UPII-SV40T male transgenic mice with 0.6% dietary FKA for 62 and 290 days starting at 28 days of age resulted in inhibition of tumor growth by weight by 27% and 59%, respectively. These results suggest that FKA may exert an *in vivo* anti-tumor effect in a time- and concentration-dependent manner. Further studies are warranted to examine the dose-dependent effects of FKA in diet, and to work on improving plasma and urine concentrations of FKA by introducing a novel FKA formulation (e.g. FKA nanoparticles or liposomes).

Our previous studies have demonstrated that FKA is a potent apoptosis inducer *in vitro* in cancer cells through both activation of proapoptotic pathways and inhibition of expression of anti-apoptotic proteins: survivin and XIAP [20–22]. Now we extend these findings to an *in vivo* setting. FKA feeding increased the number of TUNEL positive apoptotic cells in bladder tissues of the transgenic mice via up-regulating expression of a proapoptotic protein DR5 and down-regulating the expression of anti-apoptotic proteins: survivin and XIAP. Notably, there is increasing clinical interest on the use of urine-based survivin tests as an adjunct diagnostic for cystoscopy in the early detection of bladder cancer [29]. Survivin could be a potential surrogate biomarker for future chemoprevention study of bladder cancer by FKA in the clinic.

We observed a significant difference in bladder tumor growth and progression between male and female UPII-SV40T transgenic mice. The mean tumor-bearing bladder weights of male UPII-SV40T mice at 90 and 318 days of age are about 1.8 and 5.5 fold heavier, respectively, than those of females. Johnson et al [30] reported that deprivation of androgen by castration reduced tumor growth and androgen supplementation reversed the anti-tumor effect of castration via down-regulating the expression of an angiogenesis factor thrombospondin-1 in male UPII-SV40T transgenic mice. We have recently reported that flavokawains down-regulated the mRNA expression of androgen receptor in prostate cancer cells [31]. In the present study, dietary FKA was more effective in reducing tumor growth in the male transgenic mice than in females (Figure 2). In addition to its proapoptotic and anti-proliferative effect, FKA may affect angiogenesis through down-regulation of androgen receptor in bladder tumor tissues. Further studies are underway to examine the effect of FKA on androgen receptor signaling and tumor angiogenesis in both male and female UPII-SV40 transgenic mice.

Previous studies from us and other groups have demonstrated that FKA at concentrations which significantly inhibit the growth of many types of cancer cell lines has minimal effect on the growth of normal cells derived from different types of tissues, including breast, liver, prostate, fibroblast, intestine, and bone marrow [17–22, and data not shown]. In this study, non-transgenic mice that were fed with standard diet supplemented with 0.6% FKA to achieve a daily dose of about 960 mg FKA/kg body for 290 days exhibited no significant change in organ (liver, heart, kidney, and others) to body weight ratio change, body weight loss, or food and water consumption. These results suggest that dietary FKA could be safe for long-term use in cancer prevention.

In summary, low copied UPII-SV40T male mice chronologically developed urothlium-specific dysplasia, carcinoma *in situ*, and high-grade papillary UCC with or without muscle-invasion or metastasis; 77.8% of these mice develop high-grade papillary UCC at 10.5

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months of age if they live that long, and will succumb to death between 6 and 13 months. This model efficiently allows evaluation of the chemopreventive efficacy of dietary agents in bladder cancer. FKA, a kava chalcone, was excreted through the urinary tract and concentrated in urine, which led to a significant increase in the mean survival of bladder tumor bearing mice, a decrease in the occurrence of high-grade papillary UCC and a reduction in tumor size as measured by bladder weight. Dietary FKA has a satisfactory safety profile for long-term chemoprevention study. The *in vivo* mechanistic studies further supported that FKA is a robust apoptosis inducer via activation of proapoptotic pathway, and inhibition of the expression of anti-apoptotic proteins: XIAP and survivin. Survivin could be a useful surrogate biomarker for future investigative clinical trials of FKA in human urinary bladder cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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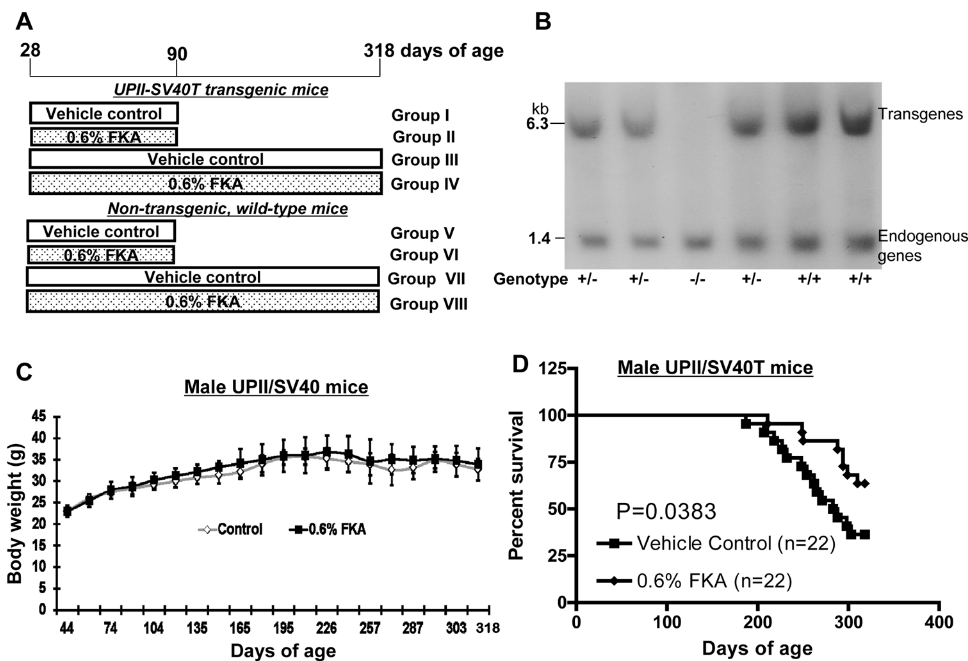

Figure 1. The effect of dietary FKA feeding on the survival of male UPII-SV40T transgenic mice

A, An experimental design to study the chemopreventive efficacy of dietary FKA feeding on bladder tumor growth and progression and the survival of bladder tumor bearing mice in UPII-SV40T transgenic mice. Low copy UPII-SV40T transgenic mice and non-transgenic wild-type control mice starting at 28 days of age were randomly assigned into different treatment groups as follows: Group I (males, 24; females, 24) and II (males, 21, females, 26), the SV40T mice were fed with control or 0.6 % FKA-supplemented [0.6 % FKA (w/w) in AIN-93M purified] diet and then sacrificed at ages 90; Groups III (males, 22; females, 25) and IV (males, 22; females, 24), the SV40T mice were fed with control or 0.6 % FKA-supplemented [0.6 % FKA (w/w) in AIN-93M purified] diet and then sacrificed at ages 318 days; Group V (males, 6; females, 5), VI (males, 6; females, 6), VII (males, 6; females, 7) and VIII (males, 6; females, 6), non-transgenic mice were treated as indicated and the then sacrificed at ages 90 and 318 days, respectively. **B,** Southern blot analysis of NcoI-digested mouse tail DNAs using UPII probe to detect a 6.3-kb transgene fragment and a 1.4-kb endogenous UPII genomic fragment. Genotypes were determined by calculating the density ratio between transgene and endogenous UPII. **C,** The comparison of mean body weights of male low copy UPII-SV40T transgenic mice which were fed with vehicle control or 0.6% FKA-supplemented diet over a period of 290 days. **D,** Survival curves of male low copy UPII-SV40T transgenic mice which received AIN-93M diet (vehicle control) or 0.6 % FKA-supplemented diet until 318 days of age.

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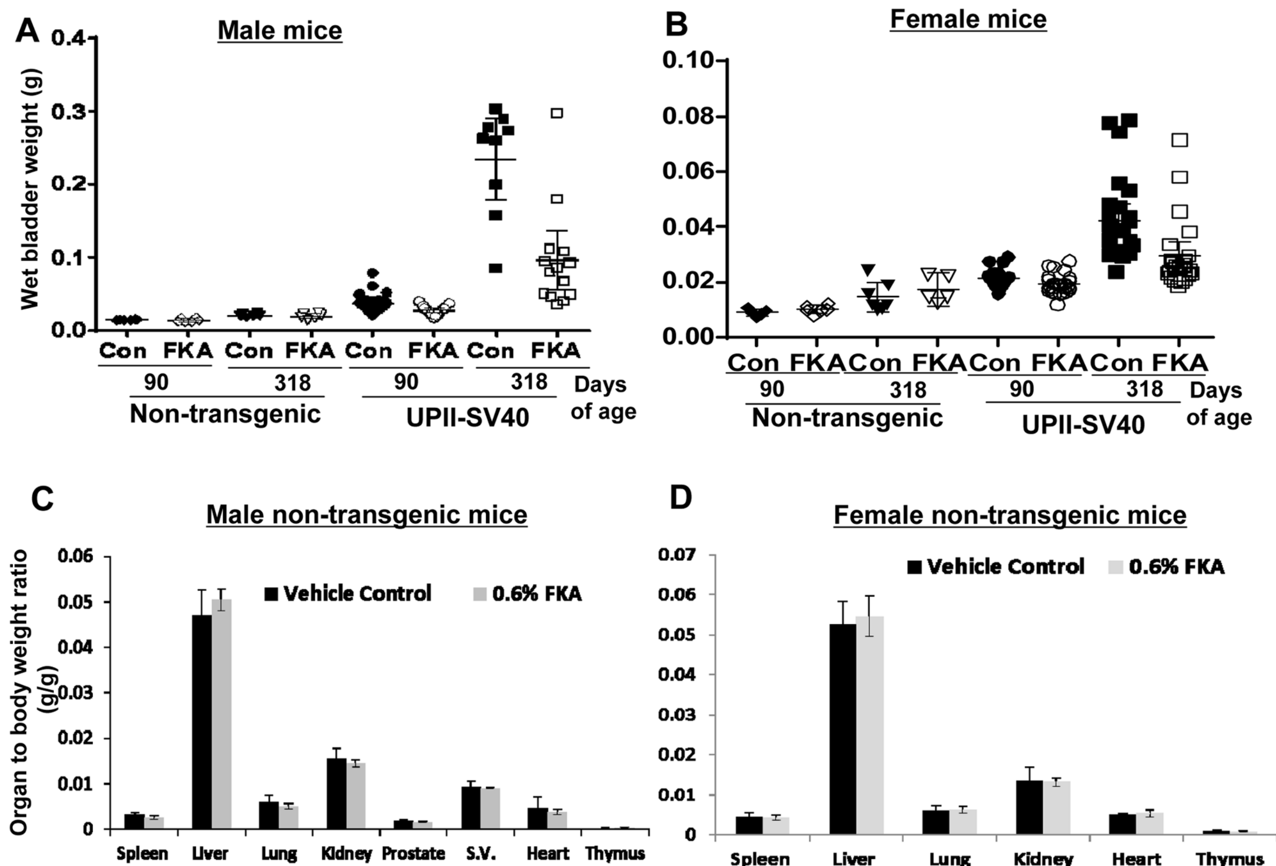

Figure 2. The effect of dietary FKA feeding on bladder weight of UPII-SV40T transgenic mice and on organ to body weight ratios of non-transgenic mice comparing gender

A, Bladder weights of male nontransgenic or UPII-SV40T transgenic mice that were fed with vehicle control or 0.6% FKA diet until 90 and 318 days of age, respectively. **B,** Bladder weights of female nontransgenic or UPII-SV40T transgenic mice that were fed with vehicle control or 0.6% FKA diet until 90 and 318 days of age, respectively. **C&D,** male and female non-transgenic mice were fed with vehicle control or 0.6% FKA diet for 290 days. Mean ratio of organ to body weight ± SD.

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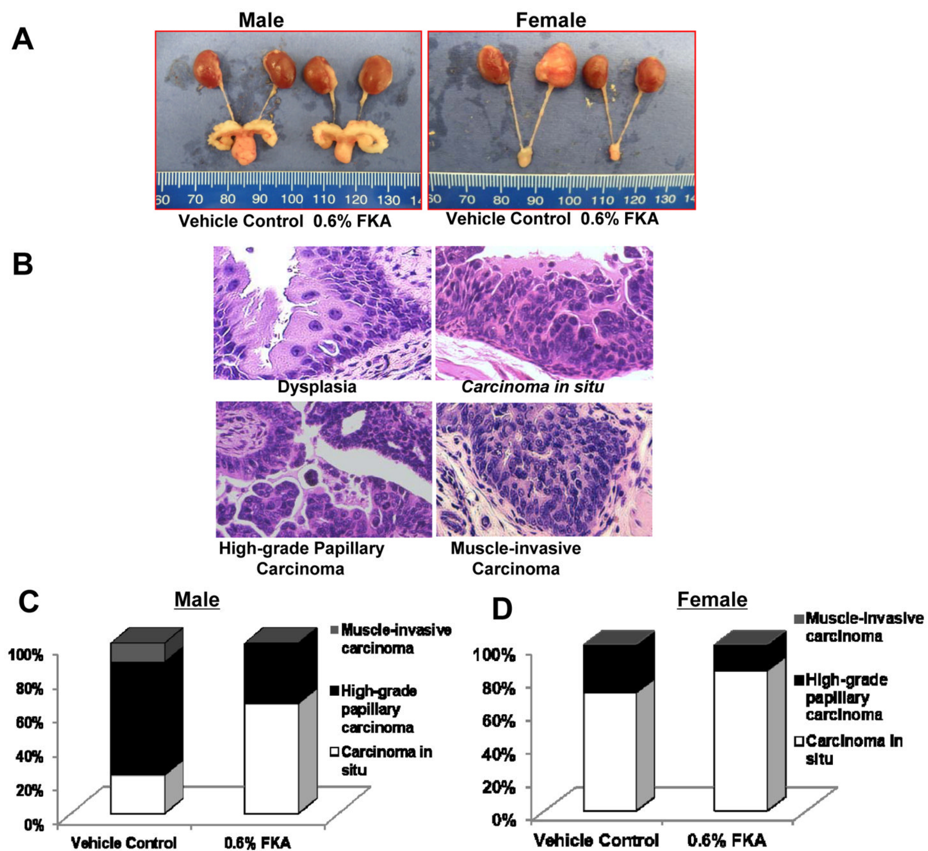

Figure 3. The effect of dietary FKA feeding for 290 days on neoplastic progression of bladder tissues at different pathological stages in UPII-SV40T transgenic mice

A, Macroscopical examination of bladders, ureters and kidneys after vehicle control or FKA feeding of low copied, male and female UPII-SV40T transgenic mice for 290 days. **B,** Representative photographs of bladder tissues at different pathological stages (normal, dysplasia, carcinoma in situ, high-grade papillary UCC, and muscle-invasive UCC) in low copied, male UPII-SV40T transgenic mice at 318 days of age, magnification X 200. **C&D,** Percentages of *carcinoma in situ*, high-grade papillary UCCs and muscle-invasive UCC after vehicle control or FKA feeding of low copied, male and female UPII-SV40T transgenic mice for 290 days.

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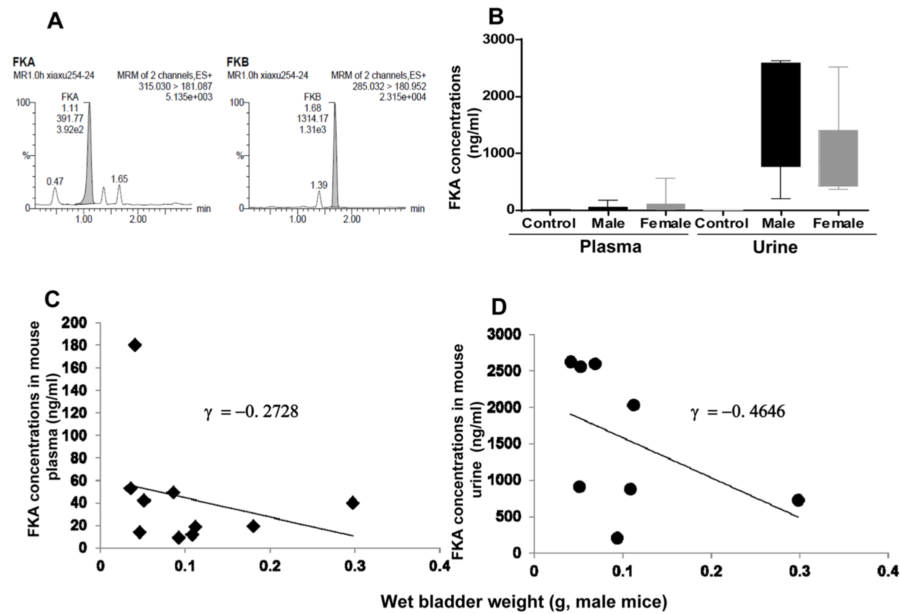

Figure 4. FKA concentrations in plasma and urine and its relationship with bladder weight of male UPII-SV40T transgenic mice

A, Chromatographic separation of FKA was achieved using an ACQUITY UPLC (Waters, UK) with a reversed-phase ACQUITY UPLCTM BEP C18 column (1.7μm, 2.1× 50mm, Waters). FKA was monitored as a precursor ion with an m/z value of 315 and a fragment ion with a value at 181.14. **A,** a representative chromatograph of the plasma level of FKA after 0.6% dietary FKA feeding for 290 days in a male UPII-SV40T transgenic mouse. Flavokawain B (FKB) was used as an internal control. **B,** The mean FKA concentrations in plasma and urine in mice fed with vehicle control or 0.6% FKA for 290 days. Bar: mean ± SD. **C,** Correlation analysis between FKA plasma concentrations and bladder weights in male low copyUPII-SV40T transgenic mice. **D,** Correlation analysis between FKA plasma concentrations and bladder weights in female low copy UPII-SV40T transgenic mice.

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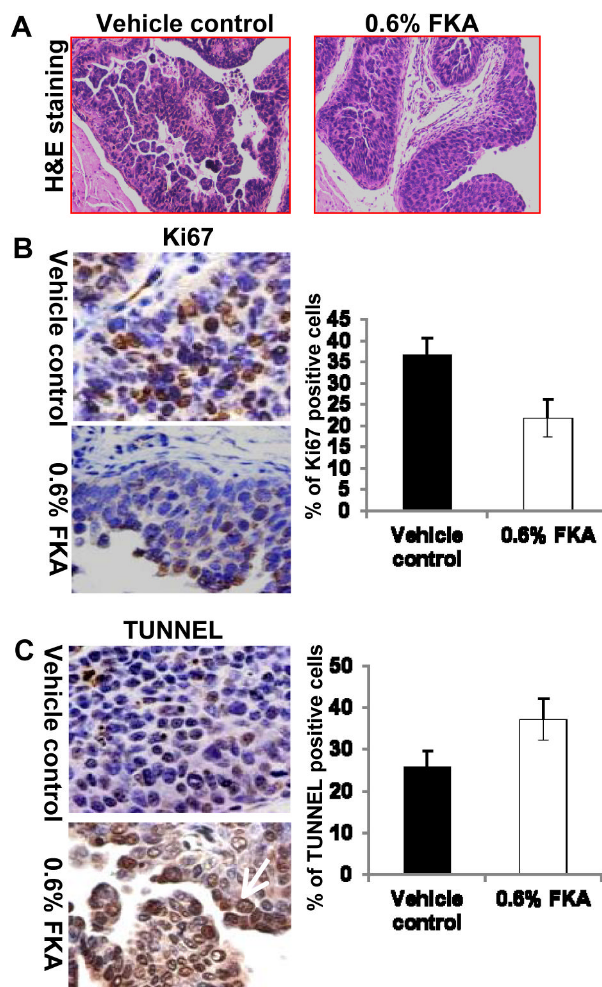

Figure 5. Antiproliferative and proapoptotic effects of dietary FKA in male UPII-SV40T transgenic mice

A, representative H&E staining of bladder tissues from vehicle control and dietary FKA fed male low copy UPII-SV40T transgenic mice. FKA feeding reduced number of high-grade papillary UCCs compared to control group. Original magnification: ×100 **B,** Immunohistochemical staining of Ki67 expression in bladder tissues. Slides were counterstained with hematoxylin and photographed using a light microscope. Original magnification: ×200. Ki67 positive cells were counted in 12 fields in each group. The percentage of Ki67 positive cells was calculated and presented as mean ± SD (the left two panels). The percentages of Ki67 positive cells are significantly lower in the FKA fed groups (n=6) than those in the vehicle control group (n=6) (Student t test, P<0.01). **C,** Representative DAB-stained tissue specimens from vehicle control and 0.6% FKA–fed group showing brown-colored TUNEL-positive cells are depicted at ×200 magnifications. Arrows, TUNEL-positive cells. Original magnification: ×200. TUNEL positive cells were counted in 12 fields in each group. The percentage of TUNEL positive cells was calculated and presented as mean ± SD (the left two panels). The percentages of TUNEL positive cells are significantly higher in the FKA fed groups (n=6) than those in the vehicle control group (n=6) (Student t test, P<0.01).

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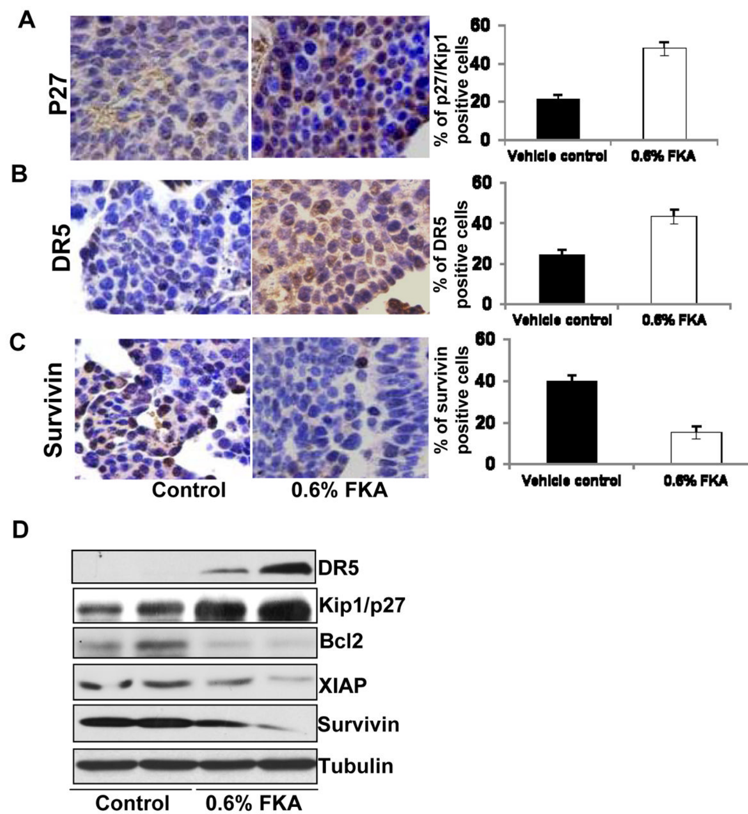

Figure 6. Effect of 290 days of dietary feeding of FKA on the expression of apoptosis and cell cycle regulators in the bladder of male low copy UPII-SV40T transgenic mice

A, B, &C, Bladder tissue specimens of six individual mice were selected from each group for immunohistochemical staining of p27/Kip1, DR5 and survivin expression. Original magnification: ×200. Positive staining cells were counted in 12 fields in each group. The percentage of positive cells was calculated and presented as mean ± SD (the left two panels). **D,** reactive protein bands were visualized by enhanced chemiluminescence detection system, and membrane was stripped and reprobed with β-actin as loading control. Representative blots of two bladder tissue specimens from each group.