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High sensitivity of an Ha-RAS transgenic model of superficial bladder cancer to metformin is associated with ~ 240-fold higher drug concentration in urine than serum

Zhongbo Liu[1], **Noriko N. Yokoyama**[1], **Chris A. Blair**[1], **Xuesen Li**[1], **Daina Avizonis**[2], **Xue-Ru Wu**[3], **Edward Uchio**[1], **Ramy Youssef**[1], **Michael McClelland**[4], **Michael Pollak**[5], and **Xiaolin Zi**[1,6,7]

[1]Department of Urology, University of California, Irvine, Orange, CA 92868, USA

[2]Metabolomics Core Facility, Goodman Cancer Research Center, McGill University, Montreal, Quebec, Canada

[3]Departments of Urology and Pathology, New York University School of Medicine and Veterans Affairs New York Harbor Healthcare System, Manhattan Campus New York, NY 10016, USA

[4]Department of Pathology and Laboratory Medicine, University of California, Irvine, Orange, CA 92868, USA

[5]Department of Oncology and Segal Cancer Center, McGill University, Montreal, Quebec, Canada

[6]Department of Pharmacology, University of California, Irvine, Orange, CA 92868, USA

[7]Chao Family Comprehensive Cancer Center, University of California, Irvine, Orange, CA 92868, USA

Abstract

While pharmacoepidemiologic and laboratory studies have supported the hypothesis that the anti-diabetic drug metformin may be useful in treating or preventing cancer, there is limited evidence to suggest which specific cancer sites may be particularly sensitive. Sensitivity likely is determined both by features of tumor pathophysiology and by pharmacokinetic factors. We used UPII mutant Ha-ras transgenic mice that develop hyperplasia and low-grade, papillary urothelial cell carcinoma to determine if metformin has activity in a model of superficial bladder cancer. Metformin significantly improved survival, reduced urinary tract obstruction, reduced bladder weight (a surrogate for tumor volume) and led to clear activation of AMP α kinase and inhibition of mTOR signaling in neoplastic tissue. We investigated the basis of the unusual sensitivity of this model to metformin, and observed that following oral dosing, urothelium is exposed to drug concentrations via the urine that are ~ 240 fold higher than those in the circulation. In addition, we observed that bladder cancer cell lines (RT4, UMUC-3 and J82) with homozygous deletion of either TSC1 or PTEN are more sensitive to metformin than those (TEU2, TCCSUP and HT1376)

*To whom all the correspondence should be addressed at Department of Urology, University of California, Irvine, 101 The City Drive South, Rt.81 Bldg.55 Rm.302, Orange CA 92868, U. S. A. Phone (714) 456-8316, Fax (714)456-1786, xzi@uci.edu.

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with wild-type TSC1 and PTEN genes. Our findings provide a strong rationale for clinical trials of oral metformin in treatment of superficial bladder cancer.

Keywords

Metformin; bladder cancer; UPII-mutant Ha-ras transgenic mice

Background

Metformin is the most widely prescribed drug for treatment of type II diabetes. Proposed mechanisms by which metformin may be useful in cancer prevention and/or treatment have been recently reviewed [1]. One possibility is that metformin acts indirectly to reduce tumor growth by altering the host endocrine/metabolic environment in a manner that reduces cancer aggressiveness. An example is metformin-induced reduction of insulin and IGF-I levels, which are mitogenic for a subset of cancers [1]. While it is clear that metformin has favorable effects on the endocrine milieu [2], it is not certain if the magnitude of these changes is sufficient to perturb tumor biology. Another possibility is that metformin may accumulate in target tissues at a sufficient concentration to act directly. The compound is known to cause energetic stress by inhibiting oxidative phosphorylation, and this may lead to death of cancer cells particularly sensitive to energetic stress, or alternatively to a cytostatic effect for the subset of cancers that react to energetic stress by reducing energetically expensive processes such as protein synthesis and proliferation. However, this mechanism can only operate if tumors are exposed to sufficient drug concentrations, and it has not been determined if this is achieved clinically following administration of conventional anti-diabetic metformin dosing.

Non-muscle invasive bladder cancer (NMIBC) is the most common neoplasm of the urinary tract. Despite current treatments such as transurethral resection, and intravesicular BCG or mitomycin C, it is associated with high risk of recurrence and/or progression to invasive and metastatic disease [3]. The need for life-long invasive surveillance and treatment places NMIBC among the most expensive cancers to treat on a per-patient basis and it represents a major public health challenge [4]. The development of a safe, low cost and orally active drug for preventing and treating recurrence and progression of NMIBC is a clear priority in urologic oncology.

Retrospective pharmaco-epidemiologic studies have suggested that treatment of diabetic patients with metformin leads to reduced burden of many cancers, relative to diabetics treated with other agents. In terms of bladder cancer specifically, Tseng et al. [5] have recently analyzed incident cases of bladder cancer in metformin users and non-users from Taiwan's National Health Insurance reimbursement databases, and reported that metformin use was associated with reduced risk of bladder cancer in patients with type 2 diabetes mellitus in a dose and time-dependent manner. In another recent retrospective and multicenter cohort study of 1117 NMIBC patients; Rieken et al. [6] reported that patients taking metformin have less disease recurrence and progression compared with patients with diabetes who did not take metformin. However, such studies have several important limitations, and should not, on their own, be regarded as sufficient to justify clinical trials

[7]. In particular, exposure to the anti-diabetic drug pioglitazone, which has been reported to be associated with increased risk of bladder cancer [8] was not excluded as a potential confounding factor in these studies.

A recent report of the first clinical trial of metformin in oncology with a survival endpoint (for pancreatic cancer) yielded disappointing results, despite prior encouraging laboratory and pharmaco-epidemiologic data [9]. This trial used a conventional antidiabetic dose of metformin, which led to serum levels in the micromolar range, raising the possibility of inadequate drug exposure for direct action, as most prior in vitro work showed activity at millimolar concentrations. As metformin is concentrated in urine [10], we hypothesized that urothelial surfaces might be exposed to considerably higher drug concentrations than other organs, and therefore be a favored site for direct action of metformin.

Over-activation of Ha-ras through ras mutation and overexpression or increased signaling by upstream receptor tyrosine kinases (RTKs) commonly occurs in NMIBC [11, 12]. The UPII mutant Ha-ras transgenic mouse model mimics human noninvasive papillary transitional urothelial cell carcinoma (UCC) in both pathology and molecular pathways [11, 12]. Male homozygous UPII mutant Ha-ras transgenic mice sequentially develop urothelial hyperplasia and non-muscle invasive papillary UCC within 6 months of age. Tumors were observed not only in the bladder, but also in the renal pelvis and ureters. From normal-appearing urothelium, homozygous UPII mutant Ha-ras transgenic mice sequentially develop simple urothelial hyperplasia, papillary hyperplasia, nodular hyperplasia and low-grade, urothelial tumors within 6 months of age. The UPII mutant Ha-ras transgenic urothelial cell carcinoma model is therefore suitable for testing the pre-clinical efficacy of metformin in preventing and treating the recurrence and progression of non-muscle invasive papillary UCC. We found that metformin significantly reduces the tumor burden in this model and extends the survival of urothelial tumor bearing mice, and prevents the development of low-grade and papillary UCC.

Materials and Methods

Mouse breeding, Southern blotting and genotyping

Heterozygous UPII- mutant Ha-ras+/− females were cross-bred with heterozygous UPII-mutant Ha-ras +/− males. Genomic DNA was isolated from tail biopsies of individual transgenic mice. DNA was digested with NcoI, resolved by gel electrophoresis, and hybridized with a probe located at the 3′ end of the UPII promoter to identify a transgene fragment (1.7 kb) and an endogenous UPII gene fragment (1.4 kb) as described by Mo et al. [12]. 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. Urothelium-specific expression of mutant Ha-ras oncogene was confirmed by RT-PCR, Western blotting, and immunohistochemical staining. It has been reported that all homozygous mice develop full-blown bladder tumors, whereas the vast majority tumors in heterozygous mice develop after age of 18 months. Therefore, homozygous male transgenic mice with hyperactivation of Ha-ras oncogene were selected for the experimental protocol.

Metformin treatment

Homozygous UPII- mutant Ha-ras mice were provided with normal drinking water or 0.5 or 1 mg/ml metformin diluted in the drinking water, starting at age of 30 days and ending at ages of 180 days. The drinking water with or without metformin was replaced twice weekly and adjusted for changes in body weight every 2 weeks. In parallel, age-matched nontransgenic mice (n = 6 mice per group) were treated similarly as overall controls for the same durations. A randomization process was used to ensure a comparable initial body weight in each group. Water consumption and animal body weight were recorded bi-weekly. Animal care and treatments were in accordance with Institutional guidelines and the approved protocol by UCI (protocol #: 2004–2540).

Histology and Immunohistochemistry

The urinary bladder, ureter and kidney were fixed in 10% neutral-buffered formalin and processed routinely for paraffin embedding, sectioning, and staining by H&E as described previously [13]. Sections of each urinary bladder tumor, ureter and kidney were histologically evaluated by a pathologist blinded to the experimental groups. Histological lesions were classified into simple hyperplasia (thickened urothelium), papillary hyperplasia (urothelium with multiple undulating folds yet no true papillary fibrovascular cores, more than seven cells in thickness), nodular hyperplasia (neovasculization and rudimentary fibrovascular cores), and low grade papillary UCC (multiple fibrovascular stalks projecting from the urothelial wall toward the lumen).

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 as described previously [13]. The sections were then incubated with mouse monoclonal anti-Ki-67 antibody (abcam, 1:800), anti-Skp2 (abcam, 1: 100) and anti-p27/Kip (BD, 1:100) for 1 h at 37°C in a humidity chamber. Isotypic IgG was used as negative controls. 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 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.

Cell lines

T24, RT4, UMUC3, 5637, TCCSUP, HT1376 and J82 cell lines were obtained from American Type Culture Collection (Manassas, VA). RT4 and T24 cells were cultured in McCoy's 5A growth medium, with 10% fetal bovine serum (FBS) added. 5637 and J82 cells were maintained in RPMI 1640 with 10% FBS. J82, UMUC3, TCCSUP, and HT1376 cell lines were cultured in EMEM medium containing 10% FBS. All cell lines used in this study were within 20 passages after receipt. The cell lines were tested and authenticated by ATCC. Normal bladder epithelial cells immortalized by viral proteins E6/E7 (TEU-2) were obtained from Dr. David J. Klumpp (Northwestern University Medical School, Chicago, Illinois).

All cells lines were also tested for known species of mycoplasma contamination using a kit from LONZA Inc. (Walkersville, MD).

MTT Assay

Cells were plated in 24-well culture plates at 2.5×10^5 cells per well into growth medium with 10% FBS. After 24 hours, the growth medium was replenished, and the appropriate concentrations of metformin were added to each well to treat cells for 3 days, as per the appended figures or tables. After treatments, MTT was added to each well at a final concentration of 1 mg/mL; the cells were then incubated at 37°C for 3 hours. The absorbance of each sample was determined at 570 nm. IC_{50s} were estimated using the best fit regression curve method in Excel. Results obtained with the MTT assay highly correlated with results obtained using cell number as an endpoint.

Western blotting analysis [14]

Urothelial cells and tumor tissues were scraped off from the urinary bladders of metformin or vehicle control-treated UPII- mutant Ha-ras mice and were homogenized in lysis buffer 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/Tris-buffered saline with Tween 20 solution. The membranes were incubated with the required primary antibody (AMPK α, TSC2, phospho-mTOR, mTOR, phospho-p70S6K, p70S6K and 4E-BP1) 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.

Urine analysis

Chemstrip 4MD urinalysis test strips were purchased from Roche Diagnostics (Laval, Quebec). Urine was collected by bladder massage from mice at 3, 4, 5 and 6 months of age into a 1.5 ml tube. The urine in the tube was thoroughly mixed and a 2 μl aliquot was pipetted onto a Chemstrip 4MD urinalysis test strip. After 60 seconds, the color change on the strip was compared to a supplied color scale. The ranges of glucose, protein, blood/hemoglobin, specific gravity, pH, Leukocytes, nitrite, ketones, uroblinogen, and bilirubin that were measured by the test strip are 2.8 – 55 mmol/L, 0 – 500 mg/dl, 0 – 250 Erythrocytes/μL, 1.000 to 1.030, 5–9, negative to ++, negative to positive, negative to +++, normal to 12 mg/dL, and negative to +++, respectively.

In vivo xenograft assay

NCR-nu/nu (nude) mice were obtained from Taconic (Germantown, NY). RT4 Cells (2×10^6) were mixed with matrigel and injected subcutaneously into the right flank of each mouse. Seven days later, the mice were randomly divided and pair matched into treatment and control groups of 5 mice each, and daily dosing was begun with normal drinking water or 0.5mg metformin diluted in drinking water. The tumor sizes were measured every 3 days and calculated by the formula: $0.5236L1(L2)2$, where *L1* is the long axis and *L2* is the short axis of the tumor.

Quantitation of Metformin

Plasma: A volume of 30 μL plasma was mixed with 30μL Liquid chromatography–mass spectrometry (LC/MS) grade acetonitrile and 35 μL of 50% Acetonitrile in water. A 5 μL aliquot of 12.5 μM Metformin-d6 HCl (CDN Isotopes, Quebec Canada) internal standard was added to each sample. Plasma samples were then centrifuged at 15 krmp for 10 minutes. at 1°C. A volume of 5 μL was injected for LC-MS/MS analysis. Urine preparation: Due to the high level of metformin in urine a 500 and 1000 fold dilutions were prepared in 50% acetonitrile/water. From these, 95μL was transferred to a fresh tube where 5 μL of 12.5 μM Metformin-d6 HCl was added. After vortexing samples were centrifuged at 15 krmp for 10 min. at 1°C. A volume of 5 μL was injected for LC-MS/MS analysis. The final urine dilutions were 526x and 1053x. The two dilution corrected measurements were averaged.

Samples were subjected to tandem mass spectrometry following similar protocol to Jagadeesh et al [15]. Briefly, data were collected using an Agilent 1290 ultra-high pressure HPLC coupled 6430 triple quadrupole mass spectrometer (Agilent Technologies). Metformin was separated from other analytes by reverse phase chromatography using a Pursuit PFP 2mm by 150mm, 3 μM column coupled to a guard column (Agilent Technologies). Separation was achieved using binary solvent gradient of solvent A consisting of 0.05% formic acid in water and solvent B consisting of acetonitrile. The gradient consisted of 3 minutes at 100% A followed by a linear gradient over 10 minutes to 70% B, followed by a 1 minute ramp to 100% B. The column was kept at 100% B for 6 minutes followed by re-equilibration at 100% A for 7 minutes. Metformin and the deuterated internal standard were detected by multiple reaction monitoring of the 130 → 71 and 136.3 → 77 quantifying transitions and the qualifying transitions 130 → 46 and 136 → 52 for metformin and Metformin-d6 HCl respectively. Ratios of endogenous metformin to the internal standard were compared to an external calibration curve with linear limits of detection from metformin standard sample concentrations of 12 nM to 80 μM (R^2=0.99975). Quality control samples run every 3 experimental samples and calibration samples run over a three-day period showed difference from expected value of <3% in the range of sample metformin levels.

Serum levels of C-peptide

Blood was collected from treated mice by cardiac puncture. Then the serum was obtained by centrifugation and stored at −80°C until analysis. Serum levels of C-peptide (an accepted surrogate marker for insulin secretion rate) [16] were measured with the RayBio mouse C-Peptide ELISA (RayBiotech) by following the kit instruction.

Statistical analysis [17]

Prism statistic software (GraphPad) was used to compute mean, standard deviations and confidence intervals of all quantitative data. Tumor, organ and body weight comparisons between vehicle control and metformin 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 metformin treatments. All statistical measures were two-sided, and P-values <0.05 were considered to be statistically significant.

Results

Effect of Metformin on the survival of male homozygous UPII- mutant Ha-ras mice

Homozygous UPII mutant Ha-ras transgenic mice that were genotyped by Southern blotting were randomized into groups given normal drinking water or 0.5 or 1 mg/ml metformin diluted in the drinking water until age of 180 days. The survival of these mice among different treatment groups was determined.

About 70% of control female UPII mutant Ha-ras mice drinking normal water (n=20) survived to 180 days, in contrast to only 30% of untreated male mice. Therefore, we used male mice for our experiments; as working with females would be have required impractical observation times. 80 % and 88.9%, respectively, of male UPII mutant Ha-ras mice provided with 0.5 or 1 mg/ml metformin in water (n=20 or 18) survived beyond 180 days of age, in contrast to 30% of the untreated male mice. Metformin intake significantly increased the absolute survival rate of male UPII mutant Ha-ras mice by 50% and 58.9%, respectively (Fig. 1A, Log-rank test, Ps<0.0001).

Metformin administered in drinking water at doses specified had no obvious toxicity or significant effect on mouse weights (supplementary Fig. 1A). Metformin also did not significantly affect average water consumption over time (Supplementary Fig. 1B).

Effect of metformin on tumor growth

Mean bladder and ureter weight were used as surrogates for tumor growth. Fig. 1B shows that the bladders, kidneys and ureters of UPII mutant Ha-ras mice in the normal water drinking group were significantly larger than those in metformin drinking groups. Metformin at concentrations of 0.5 and 1mg/ml in drinking water significantly decreased the mean bladder weights of UPII mutant Ha-ras mice by 37% and 56%, respectively [Normal drinking water *vs.* 0.5 or 1 mg/ml metformin; 25.8 ± 8.0 mg (n=6) *vs.* 16.1 ± 4.7 (n=16) or 11.5 ± 4.6 (n=16); Ps< 0.0001] at 180 days of age (Fig. 1C).

The mean ureter weights of 0.5 or 1 mg/ml metformin-treated mice are 30% and 37%, respectively, significantly less than those which drank normal water [Normal drinking water *vs.* 0.5 or 1 mg/ml metformin; 4.0 ± 1.3 mg (n=6) *vs.* 2.8 ± 0.6 (n=16) or 2.5 ± 1.3 (n=16); P= 0.0177] at 180 days of age [Fig. 1D].

Metformin inhibits the progression of hyperplasia to low-grade, papillary tumors

Fig. 2 shows that the majority (83.3%) of UPII mutant Ha-ras mice in the normal water control group exhibited multiple, urothelial tumors throughout the urinary tract, in contrast to 56.3% of mice that received metformin (P<0.05). These results provide evidence that metformin delays the progression of hyperplasia to low-grade papillary UCC.

The effect of metformin on hematuria

Microscopic hematuria is one of earliest signs for bladder and ureter lesions during the process of urothelial carcinogenesis and can be easily detected by urinalysis with dipstick [18]. We therefore examined whether appearance of hematuria in UPII mutant Ha-ras mice was influenced by metformin. 66.7%, 100%, 100%, and 100% of male UPII mutant Ha-ras mice drinking normal water showed hematuria at 3, 4, 5 and 6 months of age, respectively (Fig. 3). The appearance of hematuria was associated with initiation of papillary tumor in these mice. Mice receiving 0.5 mg/ml metformin had decreased hematuria, which was seen in 0%, 20%, 80% and 83.3 % of mice at the same time points.

Millimolar metformin concentrations are required for in vitro inhibition of urothelial cancer cell growth and the mTOR pathway

We examined the inhibitory effects of metformin on the mTOR pathway and on the proliferation of immortalized non-malignant bladder epithelial cells (TEU-2), human papillary urothelail cancer cells (RT4), muscle-invasive (T24, UMUC3, HT1376, 5637, and HT1197) bladder cancer cell lines, as well as the bone metastatic bladder cancer TCCSUP cell line, using *in vitro* cell culture systems. The IC_{50s} of metformin treatment of TEU-2, RT4, HT1376, UMUC-3, T24, J82, 5637, and TCCSUP cells for 72 hours were estimated to be 24.6, 2.7, 12.1, 2.72, 5.1, 4.6, 4.5 and 10.55 mmol/L, respectively (Fig. 4A and Table 1). Meformin preferentially reduced the proliferation of bladder cancer cell lines *versus* non-malignant TEU-2 cells. In addition, bladder cancer cell lines (RT4, UMUC-3 and J82) with homozygous deletion in either TSC1 or PTEN are more sensitive to metformin for reduction of cell viabilities than other cell lines (TEU2, TCCSUP and HT1376) (Table 1). Fig. 4B shows that metformin at millimolar concentrations activated AMPK α and inhibited phosphorylation of mTOR, 4E-BP1 and p70S6K in UMUC-3 cells. These actions are expected consequences of metformin-induced energetic stress secondary to inhibition of cellular respiration [7].

Metformin administered in a manner that inhibits urothelial neoplasia in the UPII mutant Ha-Ras model does not inhibit the growth of subcutaneous urothelial cancer xenografts

The observed antineoplastic effect of metformin in the transgenic UPII mutant Ha-Ras model was larger in magnitude than that described in most prior reports. As a first step to investigate the basis for this, we used the metformin treatment regime active in the transgenic model in a subcutaneous xenograft model (RT4). We found that metformin did not attenuate the *in vivo* growth of RT4 cells nor reduce tumor weights in this subcutaneous xenograft model (Figs. 4C and D). This raised the possibility that metformin exposure of the urothelium (via urine) may be higher than that of xenografts (via blood).

Metformin is concentrated in urine

We next examined whether the differential effect of metformin on tumor growth at two different sites (subcutaneous xenograft *vs.* bladder) is associated with different drug exposure levels. Figs. 5A and B show the typical chromatograms of three plasma (red shaded peaks) and urine (orange shaded peaks) samples from metformin treated mice with added internal isotope standard (Metformin-d6 HCl, green shaded peaks), respectively. No

significant interference was observed at the retention time of analytes or the internal isotope standard. Metformin was undetectable in plasma and urine from control treated mice. Urine concentrations of metformin were two orders of magnitude higher than serum concentrations. Mean urine concentrations of metformin in 0.5mg/ml or 1mg/ml metformin treated mice are 3377.7± 1156.45 and 6646.28± 683.83 *versus* 20.58 ± 17.39 and 27.6 ± 15.49 μM, in serum, respectively (Figs. 5C and D). In addition, there is a dose-dependent increase in urine concentration of metformin, whereas the metformin plasma concentrations are not significantly different between the two metformin dosage levels. The metformin concentrations in urine are equivalent to its effective concentrations in the *in vitro* cell culture for inhibiting the growth of bladder cancer cells and mTOR signaling as described above.

Metformin-induced decreases in proliferation are associated with down-regulation of Skp2 and up-regulation of p27 and inhibition of mTOR signaling in the bladder tumors of UPII mutant Ha-ras mice

Immunohistochemical staining demonstrates a significantly decreased number of Ki-67 positive cells in the 1 mg/ml metformin-treatment group compared to the control group. The mean percentage of Ki-67 positive cells in the control group was 31.4 ± 5.0% compared with 9.4 ± 3.5 % in 1mg/ml metformin treatment group ($P<0.01$) (Figs. 6A and B). In addition, there is a significant decrease in Skp2 positive cells and a marked increase in p27 positive cells in bladder tissue section from the metformin treated mice compared to those of control treated mice (Figs. 6A and B). These findings suggest that metformin exerts an *in vivo* anti-proliferative effect by a mechanism involving down-regulation of Skp2 and up-regulation of a CDK inhibitor p27. Western blotting analysis reveals that metformin inhibits the levels of phosphorylated mTOR and its downstream effectors: phosphorylated 4E-BP1 and p70S6K via up-regulation of phosphorylated AMPK α and TSC2 expression in bladder tissues (Fig. 6C). These results confirm that metformin attenuates mTOR signaling in bladder tumors of the UPII mutant Ha-ras mice.

Metformin in the drinking water at 0.5 or 1 mg/ml did not significantly change serum C-peptide levels, an accepted surrogate marker for insulin secretion rates, in the UPII mutant Ha-Ras mice compared to mice receiving plain water (1.43 ± 0.47 and 1.11± 0.24 *vs.* 0.92 ± 0.18 ng/mL, respectively; $P > 0.05$, student t test; Fig. 6D). This suggests that the activity of metformin in this model is more likely to be a direct effect of the drug in urothelial cells than an indirect effect related to changes in the host endocrine milieu.

Discussion

Approximately 75–85% of NMIBC recurs. This imposes a significant burden in terms of monitoring and treating this disease. Current treatments such as transurethral resection and installation of intravesicular BCG or mitomycin C are suboptimal in terms of convenience and efficacy, justifying study of leads concerning novel therapeutic approaches. Epidemiological studies have suggested that metformin reduces the occurrence and recurrences of NMIBC in patients with diabetes [5–7]. However, these reports are derived from retrospective data and require further validation. Here we provide the first

experimental evidence, using the UPII mutant Ha-ras urothelial cell carcinoma transgenic mouse model which mimics low-grade NMIBC, that metformin may be useful for management of early bladder cancer.

While there are many ongoing trials of metformin for various indications in oncology, the first randomized, placebo controlled trial with a survival endpoint yielded disappointing results [9]. It is possible that the drug may fail to accumulate to a sufficient concentration in many tissues to act directly in a clinically meaningful fashion, even if some pharmacodynamic markers are perturbed. Many mechanistic studies of metformin were performed in cell culture at media concentrations of 1 to 10 mmol/L [19, 20]. In our study, the media concentration required for inhibiting the mTOR pathway and proliferation of bladder cancer cell lines by 50% was also more than 2.5 mmol/L. However, the peak concentrations of oral metformin achieved in human and rat plasma at conventional anti-diabetic doses have been reported to be approximately 3.25 and 7.5 μg/mL, or 25 and 60 μmol/L, respectively [10, 21]. Thus, typical metformin serum concentrations achieved in diabetes treatment are 20 to 100 times lower than those that inhibit cell growth and mTOR signaling *in vitro*.

In our model, we observed that following oral dosing, urine contains more than a log higher drug concentration than serum, an observation consistent with prior pharmacokinetic studies in humans [10, 21]. This finding, together with the observation that metformin was ineffective in our subcutaneous xenograft model where cancers are exposed only the relatively low concentration present in serum, suggests that from a pharmacokinetic perspective, early urothelial cancers may be particularly interesting for clinical trials to evaluate metformin as an antineoplastic agent. However, our results from our xenograft experiment may have limitations as the tumor bearing mice can be treated for only a short period of time (14 days) due to the rapid tumor growth in this model.

Molecular mechanisms for the antineoplastic activity of metformin have been intensively studied both in *in vitro* cell culture and *in vivo* animal models. These include indirect actions, such as reduction of circulating insulin levels, as well as direct actions resulting in energetic stress and activation of AMPK in cancer cells [7, 22]. In our model, no decrease in insulin secretion (as indicated by serum levels of C-peptide) following oral metformin was detected. This demonstrates that the major suppression of the mTOR pathway seen is a consequence of direct actions of the drug on urothelial neoplasms. In addition, the *in vitro* cell culture studies showed that metformin is more effective in reducing cell viabilities of bladder cancer cell lines with Pten or TSC1 homozygous deletion than those cells harboring wild-type genes (Table 1). The PI3K/mTOR pathway is one of the most deregulated pathways in human bladder cancer via PIK3CA, loss of PTEN, TSC2, or TSC1, or constitutive activation of Ras [23–26]. Our results thus suggest that the PI3K/mTOR signaling may play a major role in early stages of disease progression. Consistently with this result, Seager et al. [27] showed that intravesical administration of an mTOR chemical inhibitor rapamycin strongly inhibited the progression from CIS to invasive bladder cancer in the bladder specific p53/Pten double knockout mouse bladder cancer model. Zhou et al. [28] also demonstrated that intraperitoneal injection of rapamycin significantly reduced tumor growth of high-grade papillary urothelial carcinoma of the bladder in the UPII Ha-ras/

SV40T double transgenic mouse model. The evidence that urothelium is exposed to urinary concentrations of metformin sufficient to inhibit mTOR signaling and progression of urothelial cancer justifies carefully designed clinical trials of the compound in early bladder cancer, even if clinical trials for other cancers where drug exposure levels are lower are disappointing.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Pollak M. Potential applications for biguanides in oncology. J Clin Invest. 2013; 123:3693–700. [PubMed: 23999444]
2. Goodwin PJ, Parulekar WR, Gelmon KA, Shepherd LE, Ligibel JA, Hershman DL, et al. Effect of metformin vs placebo on and metabolic factors in NCIC CTG MA.32. J Natl Cancer Inst. 2015; 107
3. van Rhijn BW, Burger M, Lotan Y, Solsona E, Stief CG, Sylvester RJ, et al. Recurrence and progression of disease in non-muscle-invasive bladder cancer: from epidemiology to treatment strategy. Eur Urol. 2009; 56:430–42. [PubMed: 19576682]
4. Sievert KD, Amend B, Nagele U, Schilling D, Bedke J, Horstmann M, et al. Economic aspects of bladder cancer: what are the benefits and costs? World J Urol. 2009; 27:295–300. [PubMed: 19271220]
5. Tseng CH. Diabetes and risk of bladder cancer: a study using the National Health Insurance database in Taiwan. Diabetologia. 2011; 54:2009–15. [PubMed: 21544514]
6. Rieken M, Xylinas E, Kluth L, Crivelli JJ, Chrystal J, Faison T, et al. Association of diabetes mellitus and metformin use with oncological outcomes of patients with non-muscle-invasive bladder cancer. BJU Int. 2013; 112:1105–12. [PubMed: 24053906]
7. Pollak MN. Investigating metformin for cancer prevention and treatment: the end of the beginning. Cancer Discov. 2012; 2:778–90. [PubMed: 22926251]
8. Ferwana M, Firwana B, Hasan R, Al-Mallah MH, Kim S, Montori VM, et al. Pioglitazone and risk of bladder cancer: a meta-analysis of controlled studies. Diabet Med. 2013; 30:1026–32. [PubMed: 23350856]
9. Kordes S, Pollak MN, Zwinderman AH, Mathôt RA, Weterman MJ, Beeker A, et al. Metformin in patients with advanced pancreatic cancer: a double-blind, randomised, placebo-controlled phase 2 trial. Lancet Oncol. 2015; 16:839–47. [PubMed: 26067687]
10. Graham GG, Punt J, Arora M, Day RO, Doogue MP, Duong JK, et al. Clinical pharmacokinetics of metformin. Clin Pharmacokinet. 2011; 50:81–98. [PubMed: 21241070]
11. Wu XR. Urothelial tumorigenesis: a tale of divergent pathways. Nat Rev Cancer. 2005; 5:713–25. [PubMed: 16110317]
12. Mo L, Zheng X, Huang HY, Shapiro E, Lepor H, Cordon-Cardo C, et al. Hyperactivation of Ha-ras oncogene, but not Ink4a/Arf deficiency, triggers bladder tumorigenesis. J Clin Invest. 2007; 117:314–25. [PubMed: 17256055]
13. Liu Z, Xu X, Li X, Liu S, Simoneau AR, He F, et al. Kava chalcone, flavokawain A, inhibits urothelial tumorigenesis in the UPII-SV40T transgenic mouse model. Cancer Prev Res (Phila). 2013; 6:1365–75. [PubMed: 24121102]
14. Liu Z, Li X, Simoneau AR, Jafari M, Zi X. Rhodiola rosea extracts and salidroside decrease the growth of bladder cancer cell lines via inhibition of the mTOR pathway and induction of autophagy. Mol Carcinog. 2012; 51:257–67. [PubMed: 21520297]

15. Jagadeesh B, Bharathi DV, Pankaj C, Narayana VS, Venkateswarulu V. Development and validation of highly selective and robust method for simultaneous estimation of pioglitazone, hydroxypioglitazone and metformin in human plasma by LC-MS/MS: application to a pharmacokinetic study. J Chromatogr B Analyt Technol Biomed Life Sci. 2013; 930:136–45.
16. Basu R, Breda E, Oberg AL, Powell CC, Dalla Man C, et al. Mechanisms of the age-associated deterioration in glucose tolerance: contribution of alterations in insulin secretion, action, and clearance. Diabetes. 2003; 52:1738–1748. [PubMed: 12829641]
17. Zi X, Simoneau AR. Flavokawain A, a novel chalcone from kava extract, induces apoptosis in bladder cancer cells by involvement of Bax protein-dependent and mitochondria-dependent apoptotic pathway and suppresses tumor growth in mice. Cancer Res. 2005; 65:3479–86. [PubMed: 15833884]
18. Fenner A. Bladder cancer: Microscopic haematuria associated with increased risk. Nat Rev Urol. 2014; 11:606. [PubMed: 25245242]
19. Zakikhani M, Dowling R, Fantus IG, Sonenberg N, Pollak M. Metformin is an AMP kinase-dependent growth inhibitor for breast cancer cells. Cancer Res. 2006; 66:10269–73. [PubMed: 17062558]
20. Gravel SP, Hulea L, Toban N, Birman E, Blouin MJ, Zakikhani M, et al. Serine deprivation enhances antineoplastic activity of biguanides. Cancer Res. 2014; 74:7521–33. [PubMed: 25377470]
21. Tucker GT, Casey C, Phillips PJ, Connor H, Ward JD, Woods HF. Metformin kinetics in healthy subjects and in patients with diabetes mellitus. Br J Clin Pharmacol. 1981; 12:235–46. [PubMed: 7306436]
22. Dowling RJ, Zakikhani M, Fantus IG, Pollak M, Sonenberg N. Metformin inhibits mammalian target of rapamycin-dependent translation initiation in breast cancer cells. Cancer Res. 2007; 67:10804–12. [PubMed: 18006825]
23. Cancer Genome Atlas Research Network. Comprehensive molecular characterization of urothelial bladder carcinoma. Nature. 2014; 507:315–22. [PubMed: 24476821]
24. Sjödahl G, Lauss M, Gudjonsson S, Liedberg F, Halldén C, Chebil G, et al. A systematic study of gene mutations in urothelial carcinoma; inactivating mutations in TSC2 and PIK3R1. PLoS One. 2011; 6:e18583. [PubMed: 21533174]
25. Cirone P, Andresen CJ, Eswaraka JR, Lappin PB, Bagi CM. Patient-derived xenografts reveal limits to PI3K/mTOR- and MEK-mediated inhibition of bladder cancer. Cancer Chemother Pharmacol. 2014; 73:525–38. [PubMed: 24442130]
26. Platt FM, Hurst CD, Taylor CF, Gregory WM, Harnden P, Knowles MA. Spectrum of phosphatidylinositol 3-kinase pathway gene alterations in bladder cancer. Clin Cancer Res. 2009; 15:6008–17. [PubMed: 19789314]
27. Seager CM, Puzio-Kuter AM, Patel T, Jain S, Cordon-Cardo C, Mc Kiernan J, et al. Intravesical delivery of rapamycin suppresses tumorigenesis in a mouse model of progressive bladder cancer. Cancer Prev Res (Phila). 2009; 2:1008–14. [PubMed: 19952358]
28. Zhou H, Huang HY, Shapiro E, Lepor H, Huang WC, Mohammadi M, et al. Urothelial tumor initiation requires deregulation of multiple signaling pathways: implications in target-based therapies. Carcinogenesis. 2012; 33:770–80. [PubMed: 22287562]

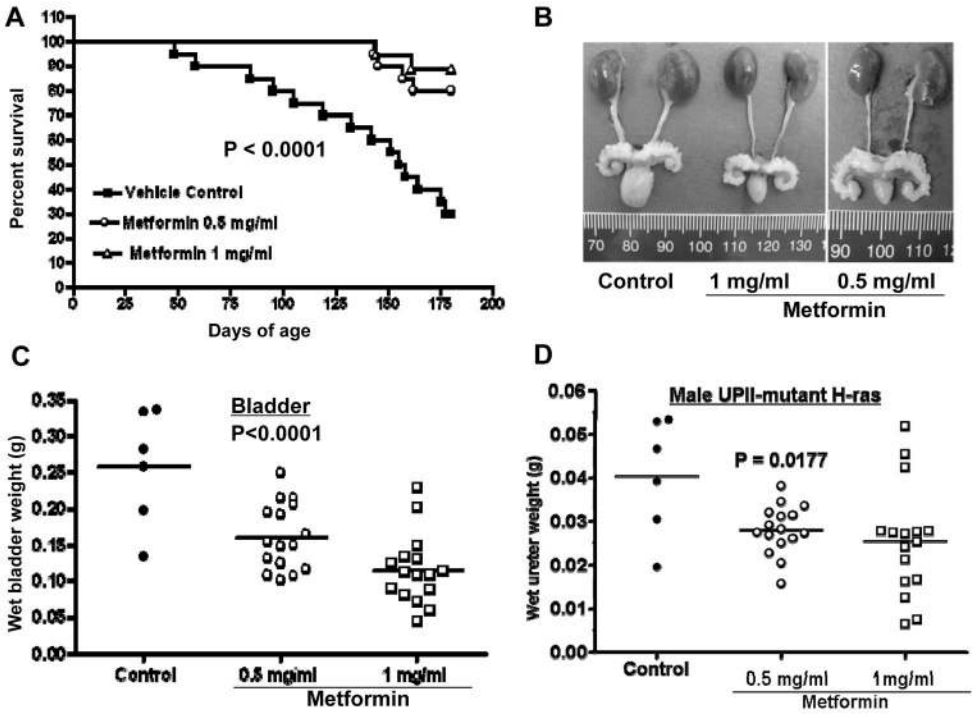

Figure 1. The effect of metformin on the survival and tumor growth of homozygous UPII-mutant Ha-ras transgenic mice

A, Survival curves of male UPII-mutant Ha-ras transgenic mice that received normal drinking water or 0.5 or 1 mg/ml metformin diluted in the drinking water until 180 days of age. **B,** Macroscopic examination of bladders, ureters and kidneys. **C &D,** Bladder and ureter weights of UPII-mutant Ha-ras transgenic mice given normal drinking water or 0.5 or 1 mg/ml metformin in the drinking water for 150 days. Mean bladder weight ± SD.

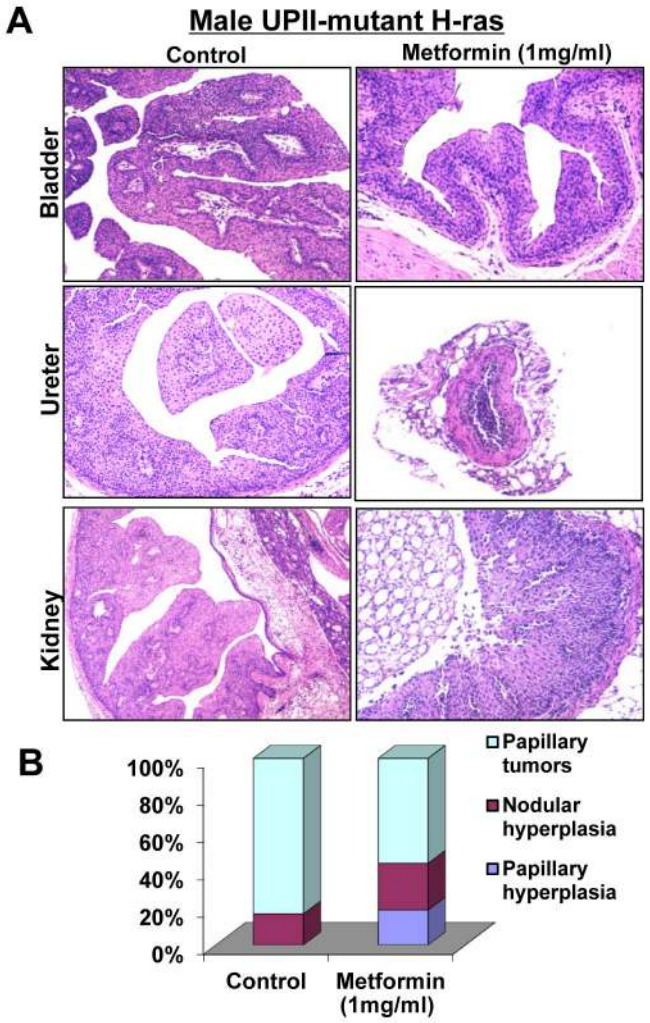

Figure 2. The effect of metfomin on the progression of hyperplasia to low-grade, papillary tumors in homozygous male UPII- mutant Ha-ras transgenic mice

A, Representative histological photographs of H&E stained bladder, ureter and kidney tissues from UPII- mutant Ha-ras transgenic mice which were treated with normal drinking water or 0.5 or 1 mg/ml metformin in the drinking water for 150 days, magnification X 200. **B,** percentages of hyperplasia and papillary tumors in the control or metformin treated UPII-mutant Ha-ras transgenic mice.

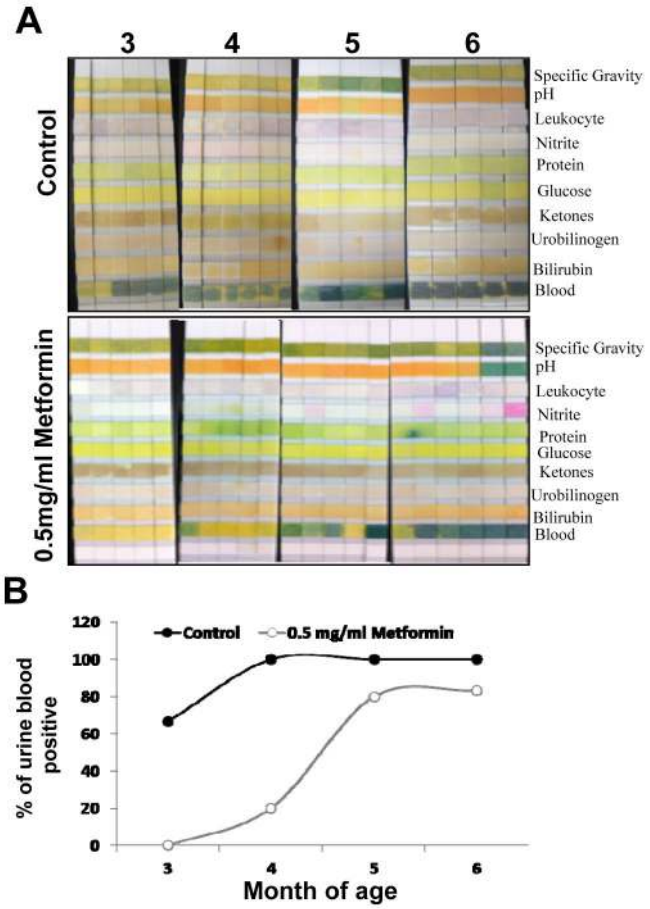

Figure 3. The effect of metformin on hematuria

A, photographs of dipsticks after loading with urine from male homozygous UPII- mutant Ha-ras transgenic mice which were administrated with normal drinking water or 0.5 mg/ml metformin in the drinking water for 90, 120, 150 and 180 days. **B,** line graphs of percentage of UPII- mutant Ha-ras transgenic mice with hematuria in the control and metformin treatment groups.

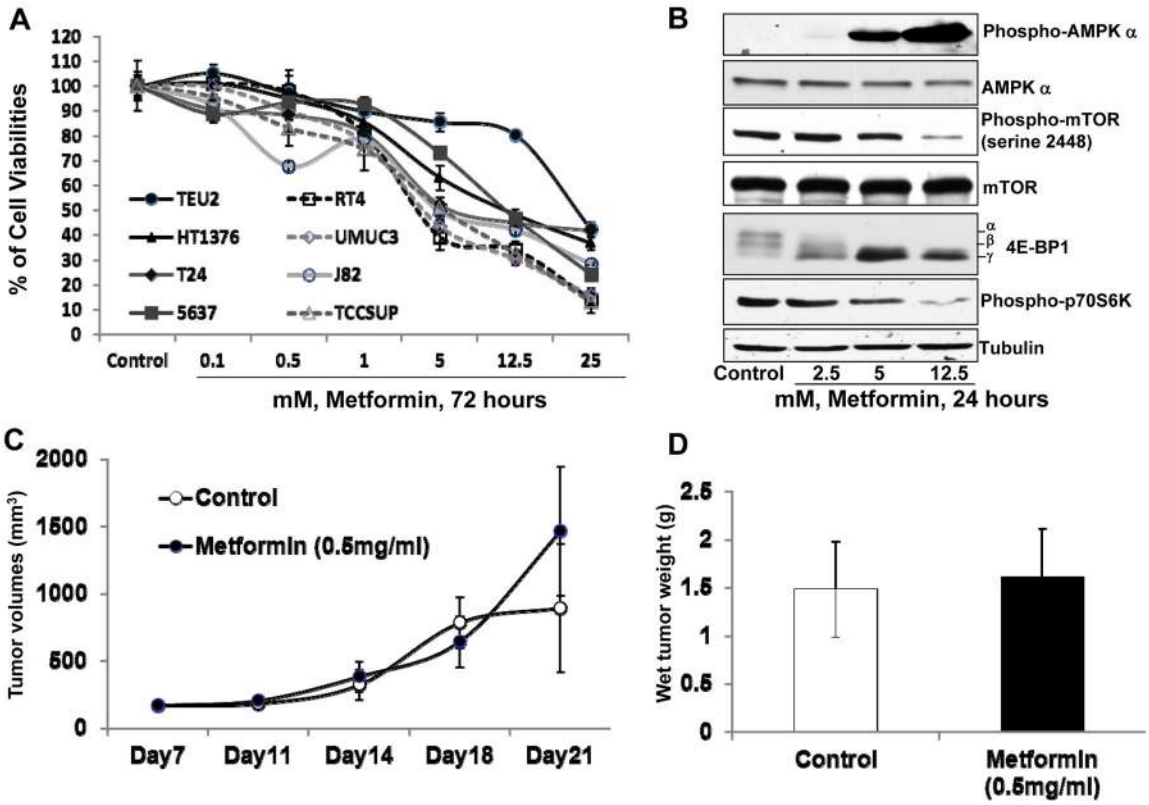

Figure 4. The effect of metformin on *in vitro* proliferation and the mTOR pathway of bladder cancer cell lines and non-malignant bladder uroepithelial cells, as well as on *in vivo* proliferation of RT4 cells in a subcutaneous xenograft model

A, cells in 24-well culture plates were treated with 0.1 % PBS or metformin at the indicated concentrations. After 72 hours of treatment, cell densities were measured by MTT assay. Each point is the mean of values from four independent plates; bars, SD. Each sample was counted in duplicate. **B,** Western blotting analysis of phospho-AMPK α AMPK α, phospho-mTOR, mTOR, phospho-p70S6K, p70S6K and 4E-BP1expression in UMUC-3 cells. Tubulin was used as a loading control. Representative blots from three replicates are presented. **C,** RT4 cells (2×10^6) in serum-free medium were mixed with equal volumes of Matrigel and injected into the right flank of NCR-nu/nu (nude) mice. After one week, the mice were randomly divided, pair-matched into treatment and control groups of 5 mice each, and daily dosing was begun with normal drinking water or 0.5 mg/ml diluted in drinking water. Tumor volumes were recorded, and presented as mean ± SE. The tumor volumes were analyzed by repeated-measures analysis of variance (ANOVA) and Ps>0.05. **D,** at the termination of the study; tumors were excised from each mouse in different groups and weighed. Columns, mean wet weight of 5 tumors from individual mice in each group; bars, SE; Student t test; P = 0.127.

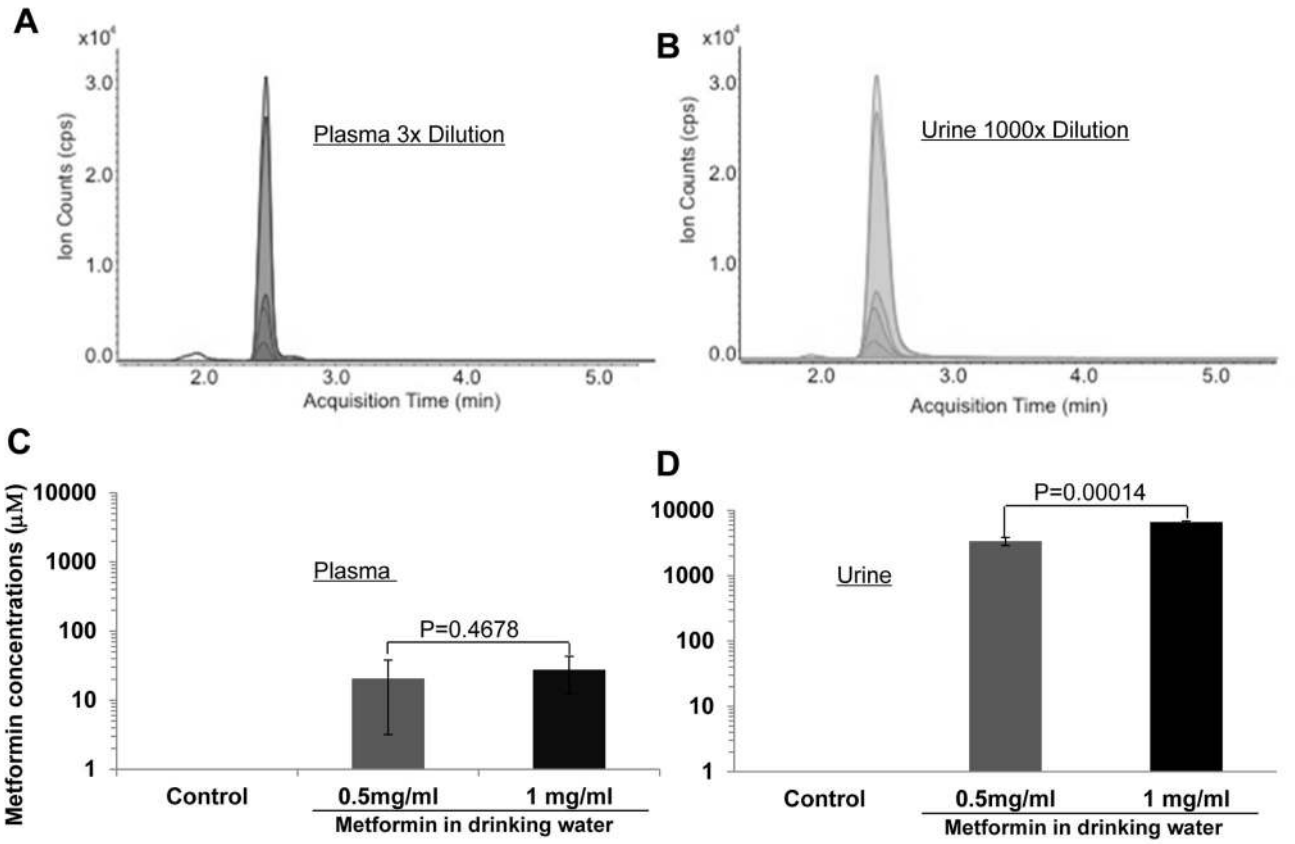

Figure 5. Metformin is highly concentrated in urine than in plasma from metformin drinking mice

A & B, representative chromatographs of the plasma and urine metformin measured by LC/MS/MS. Metformin-d6 HCl was used as an internal standard. **C & D,** the mean metformin concentrations in plasma and urine in mice fed with normal drinking water or 0.5 or 1 mg/ml metformin in drinking water. Bar, mean ± SE.

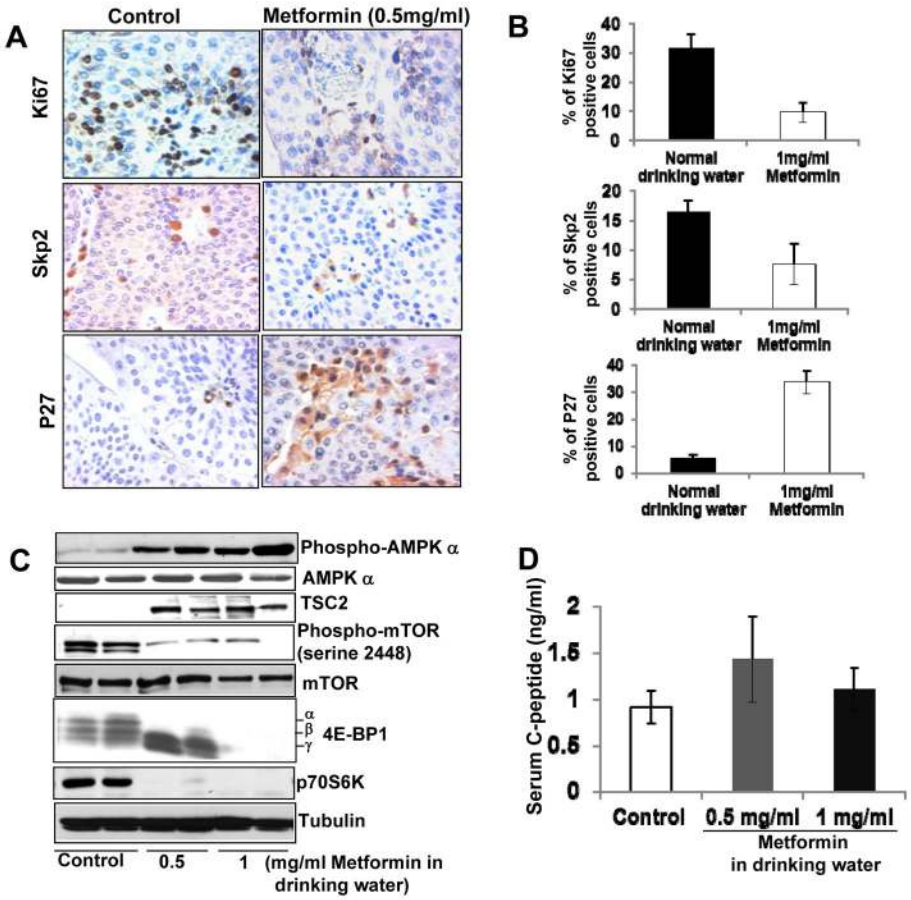

Figure 6. In vivo correlates of metformin action

A and B, Immunohistochemical staining of Ki67, Skp2 and p27 expression in bladder tissues. Slides were counterstained with hematoxylin and photographed using a light microscope. Original magnification: ×200. Ki67, Skp2 and p27 positive cells were counted in 12 fields in each group. The percentage of Ki67, Skp2 and p27 positive cells was calculated and presented as mean ± SD (the left panels). The percentages of Ki67 and Skp2 positive cells are significantly lower in the metformin treatment groups (n=6) than those in the control group (n=6) (Student t test, Ps<0.01). The percentages of p27 positive cells are significantly higher in the metformin groups (n=6) than those in the control group (n=6) (Student t test, P<0.01). **C,** bladder tissue specimens of six individual mice were selected from each group for western blotting analysis of AMPK α, phosphorylated mTOR, mTOR, 4E-BP1 and phosphorylated p70S6K expression. Reactive protein bands were visualized by enhanced chemiluminescence detection system, and membrane was stripped and reprobed with tubulin as a loading control. Representative blots of two bladder tissue specimens from each group are presented. **D,** Serum C-peptide levels, a surrogate marker for insulin secretion, in control and metformin treated mice, were measured by ELISA. Student t test, n=8, Ps >0.05.

Table 1

The IC_{50s} of metformin and tumor suppressor gene status

Cell lines	RT4	UMUC-3	J82	T24	HT1376	5637	TCCSUP	TEU2
Estimated IC_{50} (mM)	2.7	2.72	4.6	5.1	12.1	4.5	10.55	24.6
P53	WT	MT	MT	MT	MT	MT	MT	MT
TSC1	HD	WT	HD	WT	WT	WT	WT	WT
TSC2	weak	strong	Not detectable	strong	strong	strong	strong	moderate
Pten	WT	HD	HD	MT	WT	WT	WT	WT

WT = wild-type allele; MT = mutant allele; HD = homozygous deletion.