Pharmaceutical Research, Vol. 26, No. 10, October 2009 (© 2009)
DOI: 10.1007/s11095-009-9948-5

Research Paper

Pharmacokinetics and Pharmacodynamics of Broccoli Sprouts on the Suppression of Prostate Cancer in Transgenic Adenocarcinoma of Mouse Prostate (TRAMP) Mice: Implication of Induction of Nrf2, HO-1 and Apoptosis and the Suppression of Akt-dependent Kinase Pathway

Young-Sam Keum,[1] Tin Oo Khor,[1] Wen Lin,[1,2] Guoxiang Shen,[1,3] Ki Han Kwon,[1] Avantika Barve,[1,4] Wenge Li,[1] and Ah-Ng Kong[1,5]

Received November 29, 2008; accepted July 23, 2009; published online August 8, 2009

Purpose. In the present study, we have evaluated the pharmacokinetics and the *in vivo* prostate chemopreventive activity of broccoli sprouts.
Methods. The *in vivo* pharmacokinetic profiles of sulforaphane (SFN) and SFN- glutathione (GSH) conjugate in rats after oral administration of 200 mg and 500 mg broccoli sprouts were analyzed. Next, 8-week old TRAMP mice were fed with dietary broccoli sprouts at two dosages (60 and 240 mg/mouse/day) for 16 weeks, and the mice were sacrificed to examine the pharmacodynamic response on prostate tumor and some biomarkers.
Results. SFN was readily released and conjugated with GSH in the rats after oral administration of broccoli sprouts. TRAMP mice fed with 240 mg broccoli sprouts/mouse/day exhibited a significant retardation of prostate tumor growth. Western blot analysis revealed that the expression levels of Nrf2, HO-1, cleaved-Caspase-3, cleaved-PARP and Bax proteins were increased, but that of Keap1 and Bcl-XL proteins were decreased. In addition, the phosphorylation and/or the expression level of Akt and its downstream kinase and target proteins, e.g. mTOR, 4E-BP1 and cyclin D1, were reduced.
Conclusions. Our findings indicate that broccoli sprouts can serve as a good dietary source of SFN *in vivo* and that they have significant inhibitory effects on prostate tumorigenesis.

KEY WORDS: broccoli sprout; Nrf2; prostate cancer; sulforaphane; TRAMP mice.

INTRODUCTION

Prostate cancer is a multi-step molecular pathogenesis, induced by genetic and epigenetic changes that disrupt the cellular balance, such as proliferation, apoptosis, differentiation, and senescence (1). Prostate cancer currently represents a major clinical and public health challenge in the United States with a lifetime risk of being diagnosed with this disease approximately 13% (2), and, therefore, early detection and/or prevention of prostate cancer are necessary to ensure the quality of life in men (3). It has been thought that regular consumption of certain chemopreventive agents might inhibit prostate carcinogenesis in human. However, such clinical evidence has been unavailable until the completion of the Prostate Cancer Prevention Trial (PCPT), a large-scale population-based clinical trial that was launched by the National Cancer Institute (NCI) to test the efficacy of finasteride as a prostate chemopreventive agent (4). The most notable finding of this study was that the subjects who received finasteride developed a lower incidence of prostate cancer compared to those who received placebo, demonstrating for the first time that prostate cancer can indeed be inhibited by chemopreventive agent in humans. However, it was also observed that the incidence of invasive tumors was higher in the subjects that received finasteride compared to those who received the placebo. This issue makes the urologists reluctant to recommend finasteride as a possible prostate chemopreventive agent and strongly justifies additional efforts to explore safer and stronger prostate chemopreventive agent(s).

A number of case-controlled studies yield some modest support for the preventive effects of cruciferous vegetables against human prostate tumorigenesis (5). Chemoprotective effects of cruciferous vegetables are largely attributable to the dietary intake of glucosinolates, which are converted into

[1] Department of Pharmaceutics and Ernest Mario School of Pharmacy, Rutgers The State University of New Jersey, 160 Frelinghuysen Road, Piscataway, New Jersey 08854, USA.
[2] Novartis Institutes for BioMedical Research, DMPK-Translational Sciences (TS), East Hanover, New Jersey 07936, USA.
[3] Metabolism and Pharmacokinetics, Research & Development Bristol- Myers Squibb Co, Princeton, New Jersey 08543, USA.
[4] Bioanalytical R & D, Wyeth Pharmaceuticals, 401 N Middletown Rd, Pearl River, New York 10965, USA.
[5] To whom correspondence should be addressed. (e-mail: KongT@rci.rutgers.edu)

isothiocyanate metabolites in the body by the enzymatic action of plant-specific myrosinase and/or gut microflora (6). Sulforaphane (SFN; 4-methylsulfinylbutyl isothiocyanate) is a naturally-occurring isothiocyanate, which was first isolated and identified as a major principal inducer of quinone reductase (QR) in broccoli (7). Follow-up studies have demonstrated that SFN exerts significant inhibitory effects on the growth of prostate cancer cells via diverse cellular mechanisms, including the induction of apoptosis, cell-cycle arrest and phase II detoxifying enzymes, beneficial modulation of NF-κB and AP-1 and inhibition of histone deacetylase (8,9). In particular, young broccoli sprouts are an exceptionally rich source of dietary SFN in that they contain glucoraphanin, the glucosinolate precursor of SFN around 20–50 times higher than mature market-stage broccoli (10). A clinical study conducted with 200 healthy individuals in Qidong, People's Republic of China has demonstrated that daily administration of hot-water extracts of broccoli sprouts significantly reduced the level of aflatoxin-DNA adduct (aflatoxin-*N*7-guanine) and anti-phenanthrene (anti-PheT) in the urine, potentially useful biomarkers of carcinogen metabolites, implying that broccoli sprouts might have chemopreventive activities in humans (11). In the present study, we conducted *in vivo* pharmacokinetic analysis of broccoli sprouts in rats and evaluated the *in vivo* pharmacodynamic efficacy of broccoli sprouts against the prostate tumorigenesis in TRAMP mice.

MATERIALS AND METHODS

Chemicals and Reagents

SFN (purity >99%) was purchased from LKT Laboratories (St. Paul, MN). SFN-GSH (>95% purity) was chemically synthesized at Rutgers University. Other chemicals used in the study were all analytic grade unless specified.

Preparation of Broccoli Sprouts Diet

Broccoli sprouts were provided by Rick J Research Company (Columbus, OH). The source of broccoli seeds used in our experiment was Caudill Seed Co. (Louisville, KY). The seeds were sprouted with a stream of water under a high pressure (about 100+psi) and under a 400 watt HID lighting source with a constant humidity and temperature environment. After sprouting the seeds, the sprouts were harvested and dried using the same light used in the sprouting phase. The dried sprouts were then ground into a fine powder by pestle and mortar after freezing with liquid nitrogen and were mixed with AIN-76 diet pellets by the Diets Inc. (Bethelehem, PA). Both control AIN-76A diet or experimental diets, e.g. AIN-76 diets with broccoli sprouts, were stored at −20°C until the start of the experiment.

Determine the Contents of Broccoli Sprouts

To determine the contents of SFN and its glutathione conjugate SFN-GSH in the milled broccoli sprouts powder, 40 mg of the dried broccoli sprouts powder was extracted using methanol:water (70:30 v:v) using the method described by Tian *et al*. (12). Briefly, 40 mg of dried broccoli sprouts powder was sonicated for 30 min at 70°C in 0.75 ml of 70% methanol aqueous solution. After cooling in an ice bath, the supernatant of the extracts was collected by centrifugation at 15,000*g* for 15 min and then filtered through a 0.2-μm nylon filter (Waters Associates, Milford, MA). The extraction procedure was repeated three times, and the supernatants were combined. All the supernatants were transferred to a glass vial and dried under nitrogen gas. The dried samples were reconstituted in 750 μL deionized water and filtered through 0.2-μm nylon filters for analysis. An aliquot of 40 μL of the reconstituted sample was injected and subjected for LC/MS/MS analysis. MicroMass Quattro Ultima tandem mass spectrophotometer equipped with MassLynx software was used for the detection. The content of SFN precursor glucoraphanin was not determined when we prepared the diet because its authentic standard was not commercially available at the time.

Pharmacokinetic Study of Broccoli Sprouts

Male Sprague-Dawley rats (250-300 g in body weight) implanted with a jugular vein cannulation were purchased from Hilltop Animal Laboratory (Scottdale, PA). Animals were housed at the Animal Care Facility of Rutgers University, where they were acclimatized for two days and fed *ad libitum*. Rats were starved overnight before starting the experiment, and samples (300 μl) of systemic blood were obtained via jugular vein cannula at pre-dose and 0.25, 0.5, 1, 1.5, 2, 3, 4, 6, 12, and 24 h post-dose after oral gavage of broccoli sprouts. Blood was collected into heparinized syringes, immediately separated by centrifugation and stored at -20°C until further analysis. 50 μl of plasma was extracted with 400 μl methanol containing 0.5% formic acid and mixed for 4 min at room temperature. The upper layer was then transferred to a clean tube after centrifugation at 10,000*g* for 5 min and evaporated to dryness under a stream of nitrogen gas at room temperature. The obtained residue was reconstituted in 100 μl of acetonitrile/water (50:50, v/v), filtered through a 0.45 μm Nylon spin-filter (Analytical Sales, NJ) and transferred into auto-sampler vial for LC/MS/MS analysis. Similarly to above, MicroMass Quattro Ultima tandem mass spectrophotometer equipped with MassLynx software was used for the detection and quantification of the eluted analytes. The plasma concentration data were analyzed by non-compartmental pharmacokinetic analyses using WinNonlin 5.2 software (Pharsight, Mountain View, CA). The area under the plasma concentration *versus* time (AUC0−t) from time zero to the time of the last measured concentration (Clast) was calculated using the log-linear trapezoidal rule. Compartmental analysis was performed with GastroPlus 6.0 (Simulations, CA) to calculate the absorption rate constant (*ka*). The AUC0−∞ was calculated using the following equation, where the AUC0−$_{tlast}$ was calculated using the linear trapezoidal rule.

$$AUC_{0-\infty} = AUC_{0-tlast} + \frac{C_{tlast}}{k_{el}}$$

Design of TRAMP Mouse Experiment

Male TRAMP mice in pure C57BL/6 background were bred in the animal facility of Rutgers University as described

previously (13,14). All mice were maintained in a climate-controlled environment with a 12-h light/ 12-h dark cycle. After weaning at 3 to 4 weeks of age, tail DNA was clipped and used to determine the presence of the transgene T-antigen by polymerase chain reaction (PCR)-based DNA screening (15). Eight-week-old transgenic mice were randomly grouped and started to receive 3 g of control AIN76A diet pellet or 3 g of experimental AIN76A diet pellets, containing 2% (60 mg) and 8% (240 mg) broccoli sprouts. After 16 weeks, mice were sacrificed, and the weight of genitourinary apparatus (GU) consisting of the seminal vesicles, prostate and bladder was isolated for further analysis. During the course of experiment, each mouse was weighed once a week, and animal handling was conducted to monitor its general health in accordance with the established guidelines and protocols approved by Rutgers's Animal Care and Use committee.

Western Blotting and Antibodies

Prostate tumors dissected from mice in each treatment group were weighed, pooled and lyzed with RIPA buffer [50 mM NaCl, 0.5% Triton X−100, 50 mM Tris-HCl, pH 7.4, 25 mM NaF, 20 mM EGTA, 1 mM DTT, 1 mM Na3VO4, protease inhibitor cocktail tablet (Roche, Mannheim, Germany)] at a concentration of 10μg/ml for 40 min on ice, followed by centrifugation at 14,800×*g* for 15 min. The protein concentration of supernatant was measured by BCA solution (Pierce, Rockford, IL). Pooled protein samples were electrophoresed with 4–12% gradient gel and transferred onto polyvinylidene difluoride (PVDF) membrane. The membrane was blocked with 5% non-fat dry milk in 0.1% Tween-20 in PBS (PBST) for 1 h and incubated with primary antibody in 3% non-fat dry milk PBS overnight at 4°C. After three 10-min washes with 1x PBST, the membrane was then incubated with horseradish peroxidase-conjugated secondary antibody in 3% non-fat dry milk-PBS for 1 h at room temperature and washed with 1x PBST three times. The transferred proteins were visualized with the Super Signal chemiluminescent substrate (Pierce, Rockford, IL), and the intensity of the visualized bands was quantified with Quantity One software (Bio-Rad, Hercules, CA). Polyclonal antibodies against Nrf2 (57 KDa), Keap1 (70 kDa), HO-1 (32 kDa) were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Polyclonal antibodies against Bax (23 kDa), Bcl-XL(30 kDa), Akt (60 kDa), mTOR (289 kDa), 4E-BP1 (20 kDa), cleaved Caspase-3 (19 kDa), cleaved PARP (89 kDa) and phospho-specific Akt (Ser-473), mTOR (Ser- 2448), 4E-BP1 (Ser-65) antibodies were purchased from Cell Signaling Technology (Beverly, MA). Antibody against cyclin D1 (36 kDa) was purchased from Upstate Biotechnology (Lake Placid, NY).

RESULTS

Pharmacokinetic Profile of SFN and SFN-GSH After Oral Administration of Broccoli Sprouts in Rats

We have evaluated the *in vivo* pharmacokinetics of SFN and SFN-GSH conjugatein rats after oral administrations of broccoli sprouts. The milled broccoli sprouts powder used in the pharmacokinetic studies contains 104μg (0.59μmol) of SFN and 108μg (0.22 μmol) of SFN-GSH per gram of dried powder. The content of SFN precursor glucoraphanin was not determined in this study. The mean plasma-concentration time profiles of SFN in rats after oral administration of broccoli sprouts are shown in Fig. 1 (upper panel), and the summary of the pharmacokinetic parameters is shown in Table I. Likewise, the mean plasma-concentration time profiles of SFN-GSH conjugate and the summary of pharmacokinetic parameters are shown in Fig. 1 (lower panel) and Table II, respectively. The first-order absorption rate constant ka of SFN after dosing broccoli sprouts at a dose of 200 mg was 0.68 h−1 (Table I), which suggests the rapid conversion of glucoraphanine to SFN by rat gut microflora followed by fast absorption. At the doses of 200 mg and 500 mg broccoli sprouts, the Cmax of SFN was 121.3±22.6 ng/ml and 183.9±75.7 ng/ml and Tmax was about 1 h and 1.5 h in rats (Table I). The AUC of SFN was 688.5±10.6 ng*h/ml (200 mg broccoli sprouts) and 1341.1±368.4 ng*h/ml (500 mg broccoli sprouts), and T1/2 of SFN was 4.6±1.1 h (200 mg broccoli sprouts) and 3.2±0.5 h (500 mg broccoli sprouts). On the other hand, for SFN-GSH, the Cmax (451±77.3 ng/ml for 200 mg broccoli sprouts and 567±270 ng/ml for 500 mg broccoli sprouts) and AUC (1937±581 ng*h/ml at 200 mg broccoli sprouts and 5171±2068 ng*h/ml at 500 mg broccoli sprouts) (Table II) were approximately 3–4 times higher than that of SFN. The Tmax of SFN and SFN-GSH at 200 mg dose of broccoli sprouts was identical (1.0±0.5 h). Although Tmax of SFN-GSH was higher (2.6±1.6 h) than that of SFN (1.5±0.5 h) at 500 mg dose of broccoli sprouts, it was not statistically significant. Collectively, these data demonstrate that broccoli

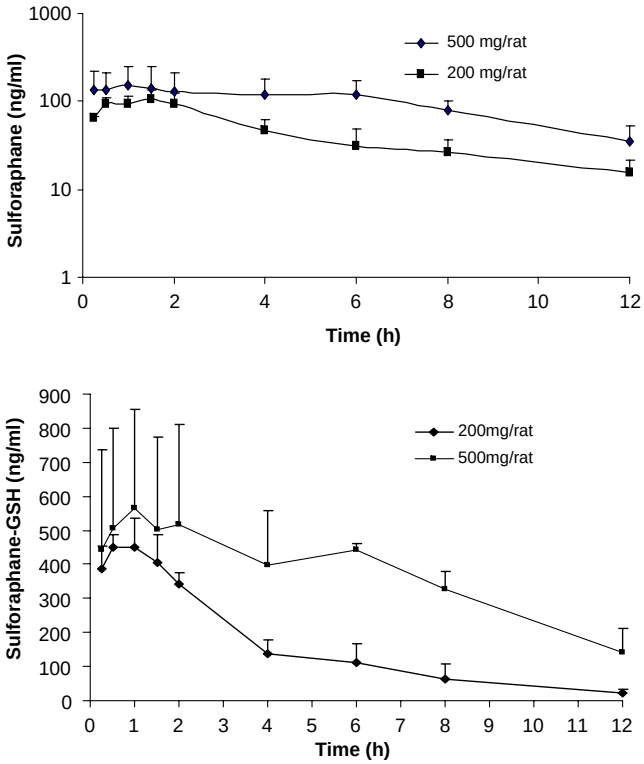

Fig. 1. The plasma concentration profile of SFN (upper panel) and SFN-GSH (lower panel) after administration of broccoli sprouts. Rats were dosed with 200 mg and 500 mg broccoli sprouts by oral gavage. The data are expressed as mean±SD, *n*=4.

Table I. Pharmacokinetic Parameters of SFN after a Single Oral Administration of 200 mg and 500 mg of Broccoli Sprouts. *N*=4

Dose (mg)	200	500
AUC0-∞(ng•hr/mL)	688.5±10.6	1341.1±368.4
AUC0-∞/dose (ng•hr/mL/ mg)	3.4±0.5	2.7±0.7
Tmax (hr)	1.0±0.5	1.5±0.5
Cmax (ng/mL)	121.3±22.6	183.9±75.7
T½ (hr)	4.6±1.1	3.2±0.5
ka (hr−1)	0.68	0.27

sprouts can provide a good dietary source of the chemopreventive SFN compound *in vivo*.

Dietary Feeding of Broccoli Sprouts Inhibits the Growth of Prostate Cancer in TRAMP Mice

In order to examine whether dietary administration by feeding of broccoli sprouts could affect the growth of prostate tumor *in vivo*, 8-week-old male TRAMP mice were fed with 3 g of control AIN-76A diet or 3 g of experimental AIN -76A diets, containing 2% and 8% broccoli sprouts per day, in which the daily amount of dietary broccoli sprouts is equivalent to 60 mg broccoli sprouts/mouse (2% broccoli sprouts group) and 240 mg broccoli sprouts/mouse (8% broccoli sprouts group), respectively (Fig. 2). During the course of study, mice were regularly inspected and weighed to monitor general health. While there was an overall gain of weight in TRAMP mice, we found no significant changes in body weight between TRAMP mice in the control diet and the experimental diet (data not shown). After 16 weeks of feeding, all the TRAMP mice were sacrificed, and the effects of broccoli sprouts on the growth of prostate tumors were evaluated by measuring the weight of GU seminal vesicle complex. As seen in Fig. 3, statistical analysis revealed that dietary administration of broccoli sprouts at a higher dose (8% broccoli sprouts diet) significantly retarded the growth of prostate tumors in TRAMP mice (p=0.006), but that of broccoli sprouts at a lower dose (2% broccoli sprouts diet) minimally suppressed it (p = 0.062), suggesting that broccoli sprouts have prostate tumor growth inhibitory effects in TRAMP mice.

Broccoli Sprouts Induce the Expression of Nrf2, HO-1 and Apoptosis in the Prostate of TRAMP Mice

Stimulation of cellular detoxification and antioxidant enzyme systems is currently regarded as a rational and effective strategy to protect the cells against inflammatory processes and/or oxidative tissue damages (16). Therefore, the induction of phase II detoxifying and antioxidant enzymes, such as glutathione S-transferases (GSTs), NAD(P)H:quinone oxidoreductase 1 (NQO1), UDP-glucuronosyltransferase (UDPGT), γ-glutamate cysteine ligase/synthetase (γ-GCL/GCS), and hemeoxygenase-1 (HO-1) have gained much attention as novel molecular targets for chemoprevention (17). Coordinated expression of these enzymes is mediated, at least in part, by a *cis*-acting element existing in the upstream promoter region of these genes, antioxidant response element (ARE) and its transactivator, NF-E2-related factor-2 (Nrf2) (18). In order to examine whether activation of Nrf2/ARE pathway might have contributed to the suppression of prostate carcinogenesis by broccoli sprouts, we have pooled the prostate samples of TRAMP mice and performed Western blotting with polyclonal Nrf2, Keap1 and HO-1 antibodies. As seen in Fig. 4, we found that the broccoli sprouts significantly induced the expression of HO-1 protein in the prostate lysates of TRAMP mice. The induction of HO-1 protein was closely correlated with the induction of Nrf2 protein as well as the suppression of Keap1 protein, implying that the induction of Nrf2 and HO-1 protein and the suppression of Keap1 proteins by broccoli sprouts might have contributed to the suppression of prostate tumor growth in TRAMP mice.

Programmed cell death (apoptosis) is a natural process that controls excessive proliferation by removing unwanted cells with harmful mutations and or cells with critical alterations in vital biological processes (19). In fact, deregulation of apoptosis is frequently observed in most types of human cancer, and a variety of chemopreventive agents are reported to exert their anti-proliferative or cytostatic effects, in part, by induction of apoptosis (20). As seen in Fig. 4, we found that administration of broccoli sprouts resulted in the activation of Caspase-3 and PARP proteins in the prostate of TRAMP mice. Activation of Caspase-3 and PARP proteins was closely associated with the induction of Bax protein and the suppression of Bcl-XL protein, both of which are important regulators of mitochondria-mediated apoptosis pathway. Our data support the notion that apoptotic induction by broccoli sprouts might be another mechanism contributing to prostate tumor growth suppression.

Dietary Broccoli Sprouts Suppress Akt-Dependent Signaling Pathway in the Prostate of TRAMP Mice

Protein synthesis is an energetically expensive and tightly regulated and controlled process at the level of initiation by modification of the eukaryotic initiation factors (eIFs), and the Akt/mTOR signaling pathway has emerged as an important regulator of protein synthesis due to its control in cap-dependent mRNA translation (21). In fact, Akt/mTOR signaling pathway is overactivated in a wide range of tumor cell types, and, therefore, blockade or interruption of this signaling cascade is currently considered as a novel strategy of chemoprevention and/or chemotherapeutics (22). As seen in Fig. 5, we found that dietary administration of broccoli sprouts at a higher dosage (8% broccoli sprouts), but not at a lower dosage (2% broccoli sprouts), considerably inhibited the phosphorylation of Akt protein in the pooled prostate

Table. II. Pharmacokinetic Parameters of SFN-GSH after a Single Oral Administration of 200 mg and 500 mg of Broccoli Sprouts. *N*=4

Dose (mg)	200	500
AUC0-∞(ng•h/ml)	1937±581	5171±2068
AUC0-∞/dose (ng•h/ml/ mg)	9.7±2.9	10.3±4.1
Tmax (h)	1.0±0.5	2.6±1.6
Cmax (ng/ml)	451±77.3	567±270
T½ (h)	2.6±1.1	3.6±0.8

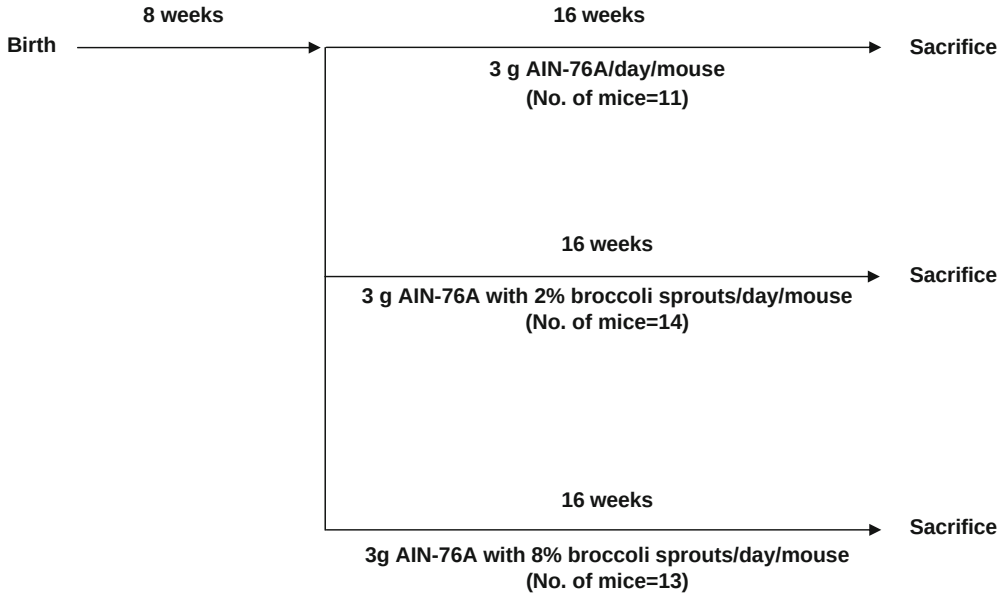

Fig. 2. Scheme of TRAMP mice experiment with the broccoli sprouts.

samples of TRAMP mice. It appears that the inhibitory effect of a higher dosage of broccoli sprouts on Akt protein activity was efficiently translated into the downstream targets because phosphorylation of mTOR and 4E-BP1, two important Akt down-stream kinase proteins, was also notably attenuated. Because Akt prevents proteosomal degradation of cyclin D1 by phosphorylating and inhibiting the activity of GSK-3β that allows cyclin D1 to accumulate (23), it is plausible to speculate that the suppression of Akt activity by broccoli sprouts might account in part for the reduced expression of cyclin D1 in the prostate of TRAMP mice. Together, our data demonstrate that notable suppression of prostate tumor growth by broccoli sprouts at the higher dosage of broccoli sprouts, but not at a lower dosage of broccoli sprouts, could be attributable, at least in part, to the inhibition of Akt-dependent signaling cascade, resulting in the suppression of protein translation (mTOR and 4E-BP1) and cell cycle progression (cyclin D1).

DISCUSSION

In the present study, we have conducted the pharmacokinetic analysis of SFN and SFN-GSH conjugate after oral

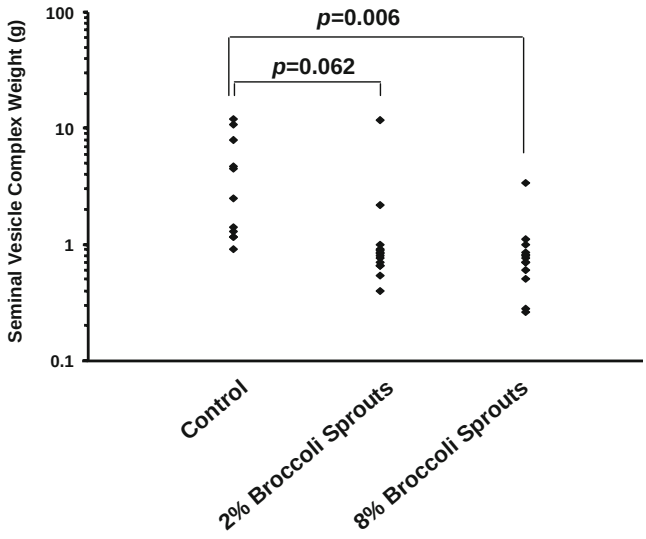

Fig. 3. Effect of dietary broccoli sprouts on the weight of seminal vesicle complex **in TRAMP mice**. Eight-week TRAMP mice started to receive a control AIN-76 diet or experimental AIN-76 diets containing 2% or 8% broccoli sprouts. After 16 weeks, mice were sacrificed, and the seminal vesicle complex was removed and weighed. Statistical analysis was conducted using Student's *t*-test.

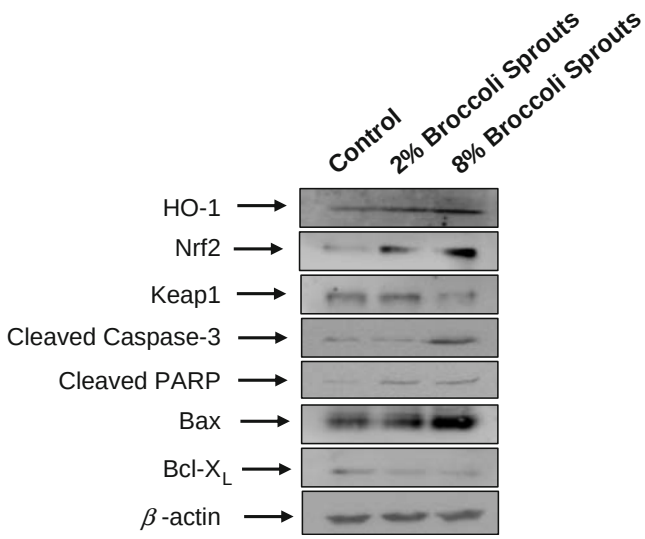

Fig. 4. Effect of dietary broccoli sprouts on the expression of HO-1, Nrf2, Keap1 **proteins and apoptotic biomarkers in the prostate tissue of TRAMP Mice.** After sacrifice, prostate tissues of TRAMP mice were pooled and subject to Western blot analysis using polyclonal antibodies against Nrf2, Keap1, HO-1, cleaved-Caspase-3, cleaved-PARP, Bax and Bcl-XL proteins (A).

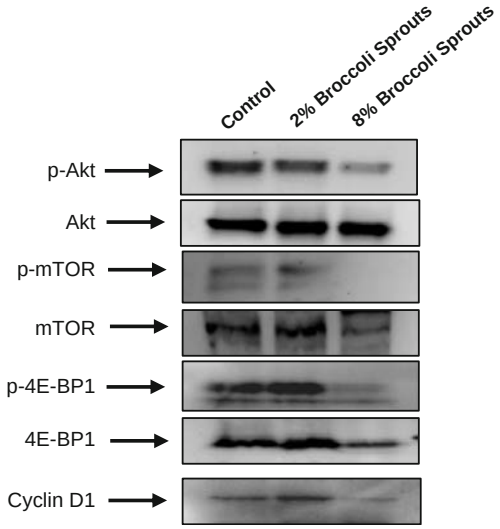

Fig. 5. Effect of dietary broccoli sprouts on the expression and/or phosphorylation **of Akt and its downstream protein targets in the prostate tissue of TRAMP mice**. After sacrifice, prostate tissues of TRAMP mice were pooled and subject to Western blot analysis using wild-type or phospho-specific Akt, mTOR, 4E-BP and wild-type cyclin D1 antibodies. Please note that Western blot analysis was conducted with the same prostate lysates used in Fig. 4.

administration of broccoli sprouts in rats. Following oral administration of 200 mg and 500 mg broccoli sprouts, peak plasma concentration of SFN and SFN-GSH conjugate occurred around 1–2 h with a *ka* of 0.68 h−1 (200 mg) and 0.27 h−1, respectively (Table I). The conjugation of SFN with GSH might have been enzymatically saturated at the high dose in rats, since the Cmax and AUC values of SFN were proportionally increased (from 121 ng/ml to 183 ng/ml and from 688 ng·hr/mL to 1341 ng·hr/mL), but that of SFN-GSH was not (from 451 ng/ml to 567 ng/ml and from 1937 ng·hr/mL to 5171 ng·hr/mL) dose-proportionally increased from 200 mg and 500 mg broccoli sprouts (Tables I and II). Although we did not measure the amount of glucoraphanin in the dried broccoli sprouts powder used in our study, there are literature reports indicating that fresh broccoli sprouts contain about 1.33 μmol/g of glucoraphanin, while various sources of broccoli sprout seeds contain 30–105 μmol/g of glucoraphanin (12)(24). Using a conservative estimation that the dried broccoli sprouts powder used in our current study would contain about 10 μmol/g of glucoraphanin, and since the content of SFN and SFN-GSH were 0.59 and 0.22 μmol/g of dried broccoli sprouts, the results are consistent with literature findings indicating that SFN primarily exists in broccoli sprouts as its precursor. The results also suggest that there was only minor conversion of glucoraphanin to SFN during the preparation process. Based on this information, we postulate that upon oral administration of the powder of broccoli sprouts, SFN would be released from broccoli sprouts through the gut microflora-mediated glucoraphanin conversion process, metabolized to form GSH conjugate and then eliminated *in vivo* according to the classical metabolic pathway for isothiocyanates, e.g. the mercapturic acid metabolism pathway. In addition, we observed that the Cmax and AUC of SFN were much lower than those of SFN-GSH, but both SFN and SFN-GSH had similar Tmax values (Tables I and II). Furthermore, we had also performed a preliminary pharmacokinetic study in which each mouse was given 200 mg doses of broccoli sprouts orally, and we found that SFN reached a Cmax value of about 68 ng/ml while the Cmax of SFN-GSH was 1,787 ng/ml (data not shown). The observation of higher concentration of SFN-GSH than SFN in the mouse after oral dosing of broccoli sprouts is consistent with the results from the rat pharmacokinetic study. Importantly, if the estimated content of glucoraphanin was 10 μmol/g in the broccoli sprouts used in the current study, then the estimated SFN-equivalent doses would be 40 and 160 ppm in 2% (60 mg/mouse/day) and 8% (240 mg/mouse/day) broccoli sprouts diet, respectively. However, since the Cmax of SFN and SFN-GSH after oral dosing of 200 mg broccoli sprouts in the mouse pilot pharmacokinetic study are comparable to our previous study of SFN in Apcmin/+ mice (25), in which the steady state concentrations of SFN and SFN-GSH were 68 and 347 ng/ml with the 600 ppm SFN diet group, these results again suggest that the glucoraphanin content in the broccoli sprouts powder would be higher than 10 μmol/g.

The experimental demonstration of exact molecular mechanisms underlying prostate carcinogenesis prevention *in vivo* by naturally-occurring or synthetic chemopreventive compounds has been hampered, largely due to the lack of the appropriate experimental animal system(s) that faithfully recapitulate human prostate carcinogenesis (26). Given the limitations of many transgenic prostate cancer mouse models, it is possible that TRAMP mice might serve as a useful model for testing the chemopreventive efficacy of broccoli sprouts since prostate cancer in TRAMP mice arises from normal cells in their natural tissue microenvironment, progresses through multiple stages, as it does in human cancer, and closely mimics human prostate carcinogenesis in terms of pathologic features and lethality (27). In addition, no exogenous chemical carcinogens and hormones are required to induce prostate carcinogenesis, since prostate carcinogenesis in TRAMP mice is mediated by the prostate-specific rat probasin promoter (−426 to +28) driving the expression of SV40-T- antigen-coding region. Furthermore, TRAMP mice reproducibly produce PIN and prostate cancer and, more importantly, will develop not only primary prostate tumors but also metastasize to other organs, such as liver, lung and lymph node. A number of promising chemopreventive substances, such as green tea polyphenols (28), nonsteroidal anti-inflammatory drugs (29), celecoxib (30), 2-methoxyestradiol (31), silibinin (32), SFN (33), PEITC and curcumin (14) as well as tocopherols (13) have been tested and shown to inhibit the growth of prostate tumors in TRAMP mice. In agreement with these observations, we found that dietary administration of broccoli sprouts at a high dosage (8% broccoli sprouts) significantly attenuated prostate tumor development in TRAMP mice, while that of broccoli sprouts with a lower dosage (2% broccoli sprouts) was minimally suppressive (Fig. 3). Using Western blot analysis, we have found that tumor growth suppression in TRAMP mice was associated not only with the activation of Nrf2/ARE-signaling cascade, i.e. the induction of Nrf2 and HO-1 proteins and the suppression of Keap1 protein, but also with the activation of mitochondria-mediated apoptotic pathway, i.e. activation of Caspase-3 and PARP proteins via an increase in Bax protein

and a decrease in Bcl-XL protein (Fig. 4). While there are no *in vivo* studies reported yet to demonstrate whether consumption of cruciferous vegetables could suppress the prostate tumorigenesis in TRAMP mice, Xiao *et al.* have reported that treatment of phenethyl isothiocyanate (PEITC) strongly induced apoptosis TRAMP-derived cell lines (TRAMP-C1 and TRAMP-C2 cells) by activating Caspase-3 and PARP proteins via an increase in pro-apoptotic protein, Bak and/or a decrease in anti-apoptotic protein, Mcl-1 and Bcl-XL (34). Because the mode of PEITC-induced apoptosis in TRAMP-C1 and -C2 cells is analogous to what we observed in the present study, we assume that TRAMP-C1 and -C2 *in vitro* cell culture system might be useful in investigating the in-depth biochemical mechanisms underlying the suppression of prostate tumor growth in TRAMP mice by broccoli sprouts *in vivo*. Most recently, we have found that pure compounds of PEITC and curcumin effectively inhibited prostate tumorigenesis in TRAMP mice (14). Similarly Singh *et al*. recently showed that pure compound SFN suppressed prostate tumorigenesis in TRAMP mice, corroborating our current study (35).

Increasing evidence points out that the Akt-dependent signaling regulatory proteins are critical regulators of prostate tumorigenesis *in vivo*. For example, Chen *et al.* have shown that the deficiency of Akt1 suppressed the development of prostate tumor neoplasia in Pten (+/−) mice (36). Trotman *et al.* have shown that prostate tumorigenesis driven in Pten-heterozygous mutant mice was significantly accelerated by a loss of PML suppressor via stimulation of Akt phosphorylation and/or suppression of Akt antagonizing effects by its phosphatase protein, PPa2 (37). Gao *et al.* have reported that Akt phosphorylation is highly upregulated and critical in the development of prostate cancer in Nkx3.1/Pten knock-out mice (38). In spite of these results, there have been no studies conducted to implicate the modulation of Akt signaling pathway in the prevention of prostate cancer by cruciferous vegetables or by any other chemopreventive isothiocyanates *in vivo*. Our study presents the first experimental evidence that the inhibition of Akt pathway might be an important cellular mechanism for prostate tumor growth suppression in TRAMP mice by broccoli sprouts. In addition, our data provide the possibility that not only the induction of Nrf2-dependent and apoptotic pathway, but also the inhibition of Akt, is required to significantly repress the prostate tumor growth in TRAMP mice. This is because the suppression of prostate tumor growth in TRAMP mice was statistically significant at a higher dosage of broccoli sprouts, but not at a lower dosage (Fig. 3) and because the inhibition of Akt and its downstream kinases (mTOR and 4E- BP) or target protein (cyclin D1) was evident only in the prostate of TRAMP mice fed with the higher dosage of broccoli sprouts (Fig. 5).

Collectively, in this study we show that broccoli sprouts can serve as a good source of naturally-occurring chemopreventive SFN. In addition, we provide evidence that a dietary administration of broccoli sprouts exhibits significant inhibitory effects on the growth of prostate tumors in TRAMP mice. Furthermore, we show that the induction of Nrf2/ARE-signaling pathway and pro-apoptotic proteins, as well as the suppression of Akt/mTOR cascade, might be implicated in the suppression of prostate tumor growth in TRAMP mice by broccoli sprouts *in vivo*.

ACKNOWLEDGEMENT

We thank Mr. Richard Jarrell for providing us with the broccoli sprouts. We thank all the members in Dr. Ah-Ng "Tony" Kong's lab for the help in discussion and preparation of this manuscript. This work was supported in part by the National Institute of Health Grant R01-CA118947 and Institutional Funds.

REFERENCES

1. Vogelstein B, Kinzler KW. Cancer genes and the pathways they control. Nat Med. 2004;10:789–99.
2. Eastham JA. Prostate-specific antigen doubling time as a prognostic marker in prostate cancer. Nat Clin Pract Urol. 2005;2:482–91.
3. Klein EA. Can prostate cancer be prevented? Nat Clin Pract Urol. 2005;2:24–31.
4. Goetzland MA, Holzbeierlein JM. Finasteride as a chemopreventive agent in prostate cancer: impact of the PCPT on urologic practice. Nat Clin Pract Urol. 2006;3:422–9.
5. Kristaland AR, Lampe JW. Brassica vegetables and prostate cancer risk: a review of the epidemiological evidence. Nutr Cancer. 2002;42:1–9.
6. Zhang Y. Cancer-preventive isothiocyanates: measurement of human exposure and mechanism of action. Mutat Res. 2004;555: 173–90.
7. Zhang Y, Talalay P, Cho CG, Posner GH. A major inducer of anticarcinogenic protective enzymes from broccoli: isolation and elucidation of structure. Proc Natl Acad Sci U S A. 1992;89: 2399–403.
8. Keum YS, Jeong WS, Kong AN. Chemoprevention by isothiocyanates and their underlying molecular signaling mechanisms. Mutat Res. 2004;555:191–202.
9. Myzakand MC, Dashwood RH. Histone deacetylases as targets for dietary cancer preventive agents: lessons learned with butyrate, diallyl disulfide, and sulforaphane. Curr Drug Targets. 2006;7:443–52.
10. Fahey JW, Zhang Y, Talalay P. Broccoli sprouts: an exceptionally rich source of inducers of enzymes that protect against chemical carcinogens. Proc Natl Acad Sci U S A. 1997;94:10367–72.
11. Kensler TW, Chen JG, Egner PA, Fahey JW, Jacobson LP, Stephenson KK, *et al.* Effects of glucosinolate-rich broccoli sprouts on urinary levels of aflatoxin-DNA adducts and phenanthrene tetraols in a randomized clinical trial in He Zuo township, Qidong People's Republic of China. Cancer Epidemiol Biomarkers Prev. 2005;14:2605–13.
12. Tian Q, Rosselot RA, Schwartz SJ. Quantitative determination of intact glucosinolates in broccoli, broccoli sprouts, Brussels sprouts, and cauliflower by high-performance liquid chromatography-electrospray ionization-tandem mass spectrometry. Anal Biochem. 2005;343:93–9.
13. Barve A, Khor TO, Nair S, Reuhl K, Suh N, Reddy B, *et al.* Gamma-tocopherol-enriched mixed tocopherol diet inhibits prostate carcinogenesis in TRAMP mice. Int J Cancer. 2009;124:1693–9.
14. Barve A, Khor TO, Hao X, Keum YS, Yang CS, Reddy B, *et al.* Murine prostate cancer inhibition by dietary phytochemicals—curcumin and phenyethylisothiocyanate. Pharm Res. 2008;25:2181–9.
15. Greenberg NM, DeMayo FJ, Sheppard PC, Barrios R, Lebovitz R, Finegold M, *et al.* The rat probasin gene promoter directs hormonally and developmentally regulated expression of a heterologous gene specifically to the prostate in transgenic mice. Mol Endocrinol. 1994;8:230–9.
16. T.W. Kensler, N. Wakabayashi, and S. Biswal. Cell Survival Responses to Environmental Stresses Via the Keap1-Nrf2-ARE Pathway. Annu Rev Pharmacol Toxicol (2006).
17. Jeong WS, Jun M, Kong AN. Nrf2: a potential molecular target for cancer chemoprevention by natural compounds. Antioxid Redox Signal. 2006;8:99–106.

18. Shen G, Jeong WS, Hu R, Kong AN. Regulation of Nrf2, NF-kappaB, and AP-1 signaling pathways by chemopreventive agents. Antioxid Redox Signal. 2005;7:1648–63.
19. Hanahanand D, Weinberg RA. The hallmarks of cancer. Cell. 2000;100:57–70.
20. Danialand NN, Korsmeyer SJ. Cell death: critical control points. Cell. 2004;116:205–19.
21. Mamane Y, Petroulakis E, LeBacquer O, Sonenberg N. mTOR, translation initiation and cancer. Oncogene. 2006;25:6416–22.
22. Sabatini DM. mTOR and cancer: insights into a complex relationship. Nat Rev Cancer. 2006;6:729–34.
23. Diehl JA, Cheng M, Roussel MF, Sherr CJ. Glycogen synthase kinase- 3beta regulates cyclin D1 proteolysis and subcellular localization. Genes Dev. 1998;12:3499–511.
24. West LG, Meyer KA, Balch BA, Rossi FJ, Schultz MR, Haas GW. Glucoraphanin and 4-hydroxyglucobrassicin contents in seeds of 59 cultivars of broccoli, raab, kohlrabi, radish, cauliflower, brussels sprouts, kale, and cabbage. J Agric Food Chem. 2004;52:916–26.
25. Shen G, Khor TO, Hu R, Yu S, Nair S, Ho CT, *et al*. Chemoprevention of familial adenomatous polyposis by natural dietary compounds sulforaphane and dibenzoylmethane alone and in combination in ApcMin/+ mouse. Cancer Res. 2007;67:9937–44.
26. Lamband DJ, Zhang L. Challenges in prostate cancer research: animal models for nutritional studies of chemoprevention and disease progression. J Nutr. 2005;135:3009S–15.
27. Klein RD. The use of genetically engineered mouse models of prostate cancer for nutrition and cancer chemoprevention research. Mutat Res. 2005;576:111–19.
28. Adhami VM, Siddiqui IA, Ahmad N, Gupta S, Mukhtar H. Oral consumption of green tea polyphenols inhibits insulin-like growth factor-I- induced signaling in an autochthonous mouse model of prostate cancer. Cancer Res. 2004;64:8715–22.
29. Narayanan BA, Narayanan NK, Pittman B, Reddy BS. Regression of mouse prostatic intraepithelial neoplasia by nonsteroidal anti-inflammatory drugs in the transgenic adenocarcinoma mouse prostate model. Clin Cancer Res. 2004;10:7727–37.
30. Gupta S, Adhami VM, Subbarayan M, MacLennan GT, Lewin JS, Hafeli UO, *et al*. Suppression of prostate carcinogenesis by dietary supplementation of celecoxib in transgenic adenocarcinoma of the mouse prostate model. Cancer Res. 2004;64:3334–43.
31. Garcia GE, Wisniewski HG, Lucia MS, Arevalo N, Slaga TJ, Kraft SL, *et al*. 2-Methoxyestradiol inhibits prostate tumor development in transgenic adenocarcinoma of mouse prostate: role of tumor necrosis factor-alpha-stimulated gene 6. Clin Cancer Res. 2006;12:980–8.
32. Raina K, Blouin MJ, Singh RP, Majeed N, Deep G, Varghese L, *et al*. Dietary feeding of silibinin inhibits prostate tumor growth and progression in transgenic adenocarcinoma of the mouse prostate model. Cancer Res. 2007;67:11083–91.
33. Singh SV, Warin R, Xiao D, Powolny AA, Stan SD, Arlotti JA, *et al*. Sulforaphane inhibits prostate carcinogenesis and pulmonary metastasis in TRAMP mice in association with increased cytotoxicity of natural killer cells. Cancer Res. 2009;69:2117–25.
34. Xiao D, Zeng Y, Choi S, Lew KL, Nelson JB, Singh SV. Caspase-dependent apoptosis induction by phenethyl isothiocyanate, a cruciferous vegetable-derived cancer chemopreventive agent, is mediated by Bak and Bax. Clin Cancer Res. 2005;11:2670–9.
35. Singh SV, Warin R, Xiao D, Powolny AA, Stan SD, Arlotti JA, *et al*. Sulforaphane inhibits prostate carcinogenesis and pulmonary metastasis in TRAMP mice in association with increased cytotoxicity of natural killer cells. Cancer Res. 2009;69:2117–25. Epub 2009 Feb 2117.
36. Chen ML, Xu PZ, Peng XD, Chen WS, Guzman G, Yang X, *et al*. The deficiency of Akt1 is sufficient to suppress tumor development in Pten+/− mice. Genes Dev. 2006;20:1569–74.
37. Trotman LC, Alimonti A, Scaglioni PP, Koutcher JA, Cordon-Cardo C, Pandolfi PP. Identification of a tumour suppressor network opposing nuclear Akt function. Nature. 2006;441:523–7.
38. Gao H, Ouyang X, Banach-Petrosky WA, Gerald WL, Shen MM, Abate-Shen C. Combinatorial activities of Akt and B-Raf/Erk signaling in a mouse model of androgen-independent prostate cancer. Proc Natl Acad Sci U S A. 2006;103:14477–82.