

Research Article

See related commentary by Sporn and Liby, p. 384

Effects of Lycopene on Protein Expression in Human Primary Prostatic Epithelial Cells

Xi Qiu¹, Yang Yuan¹, Avani Vaishnav², Michael A. Tessel², Larisa Nonn², and Richard B. van Breemen¹

Abstract

Clinical trials and animal studies have suggested that lycopene, the red carotenoid found in tomatoes, might be useful for the prevention of prostate cancer in the diet or as a dietary supplement through a variety of chemoprevention mechanisms. As most mechanism of action studies have used prostate cancer cells or males with existing prostate cancer, we investigated the effects of lycopene on protein expression in human primary prostatic epithelial cells. After treatment with lycopene at a physiologically relevant concentration (2 $\mu\text{mol/L}$) or placebo for 48 hours, the primary prostatic epithelial cells were lysed and fractionated using centrifugation into cytosolic/membrane and nuclear fractions. Proteins from lycopene-treated and placebo-treated cells were trypsinized and derivatized for quantitative proteomics using isobaric tags for relative and absolute quantitation (iTRAQ) reagent. Peptides were analyzed using two-dimensional microcapillary high-performance liquid chromatography-tandem mass spectrometry to identify proteins that were significantly upregulated or downregulated following lycopene exposure. Proteins that were most affected by lycopene were those involved in antioxidant responses, cytoprotection, apoptosis, growth inhibition, androgen receptor signaling, and the Akt/mTOR cascade. These data are consistent with previous studies suggesting that lycopene can prevent cancer in human prostatic epithelial cells at the stages of cancer initiation, promotion, and/or progression. *Cancer Prev Res*; 6(5); 419–27. ©2013 AACR.

Introduction

Prostate cancer is the second leading cause of cancer-related deaths in men in the United States with an average age of 67 years at diagnosis, and 241,740 new cases and 28,170 deaths are anticipated for 2012 (1). Carcinogenesis is a multistep process that is characterized by alterations in pathways involving cellular proliferation, differentiation, apoptosis, and senescence (2). Preneoplastic lesions in prostate tissue represent intermediate stages between normal and malignant epithelium, and as these lesions may appear decades before diagnosis of prostate cancer, the slow progression of prostate cancer might provide an opportunity for intervention and chemoprevention.

Although lacking provitamin A activity, lycopene is the most abundant carotenoid in tomatoes and is the most potent antioxidant among the naturally occurring carotenoids (3). Studies in humans and animal models indicate

that blood lycopene levels and intake of tomato-derived products are inversely correlated with prostate cancer risk (4). Clinical trials have shown that dietary tomato sauce or lycopene supplementation in men increases lycopene levels in prostate tissue and serum, reduces DNA oxidation in prostate tissue, and lowers serum prostate-specific antigen (5, 6).

Postulated mechanisms of cancer chemoprevention by lycopene include inhibition of 5-lipoxygenase (7), regulation of the IGF-1/IGFBP-3 system (8), modulation of immune function (9), upregulation of phase II cytoprotective enzymes (10), inhibition of growth, and induction of differentiation in cancer cells by modulating the expression of cell-cycle regulatory proteins (11), prevention of oxidative DNA damage (12), upregulation of Cx43 and increased gap junction intercellular communication (13), balancing redox signaling (14), and inhibition of inflammation and androgen receptor signaling (15). Evidence for these mechanisms of lycopene action has usually been obtained by studying a single pathway instead of looking at multiple mechanisms and has involved studies of cancer cells or preexisting cancer instead of primary cells or healthy individuals.

Previously, we used iCAT quantitative proteomics to examine how lycopene alters protein expression in the LNCaP human prostate cancer cell line (16) and found that phase II enzymes involved in protecting cells from oxidative stress and detoxifying electrophilic xenobiotics were upregulated by lycopene. Here, more sensitive iTRAQ

Authors' Affiliations: ¹Department of Medicinal Chemistry and Pharmacognosy, University of Illinois College of Pharmacy; and ²Department of Pathology, University of Illinois College of Medicine, Chicago, Illinois

Note: Supplementary data for this article are available at Cancer Prevention Research Online (<http://cancerprevres.aacrjournals.org>).

Corresponding Author: Richard B. van Breemen, Department of Medicinal Chemistry and Pharmacognosy, University of Illinois College of Pharmacy, 833 S. Wood Street, Chicago, IL 60612. Phone: 312-996-9353; Fax: 312-996-7107; E-mail: Breemen@uic.edu

doi: 10.1158/1940-6207.CAPR-12-0364

©2013 American Association for Cancer Research.

proteomics was used to determine how cellular protein levels are altered upon exposure of primary human prostatic epithelial (PrE) cells to physiologically relevant concentrations of lycopene. The use of healthy prostate epithelial cells instead of prostate cancer cells is ideal for prostate cancer chemoprevention studies because most prostate cancers are of epithelial origin (17, 18).

Materials and Methods

Materials

Prostate epithelial growth medium was purchased from Lonza. Water dispersible beadlets containing 10% (w/w) lycopene and identical placebo beadlets were gifts from DSM. Protease inhibitor cocktail was purchased from Sigma-Aldrich. iTRAQ reagents were purchased from AB Sciex, and trypsin was purchased from Promega. The Bio-Rad protein assay kit was purchased from Bio-Rad. Monoclonal antibodies for Western blots were purchased from Cell Signaling Technology except for Rad23b (hHR23b), which was purchased from Abcam. Buffers, high-performance liquid chromatography (HPLC)-grade solvents and all other chemicals and were purchased from Thermo Fisher.

Human primary prostatic epithelial cell culture

Prostatic epithelial (PrE) cell cultures were derived from histologically benign areas of the prostate peripheral zone from radical prostatectomy specimens of patients who had received no prior chemical, hormonal, or radiotherapy as described previously (19). Briefly, the prostate tissues were minced and digested overnight with collagenase. The digested tissues were plated onto collagen-coated dishes, and PrE cells were cultured in prostate epithelial growth media. PrE cells from 3 different patients were used for this study (PrE-1, PrE-2, PrE-3).

When the cells reached 70% confluence, they were aliquoted into 25 to 50 freezer vials and cryopreserved in liquid nitrogen storage. Individual frozen aliquots of PrE cells were thawed for each set of experiments, and cell cultures were incubated in a humidified incubator with 95% air/5% CO₂ at 37°C in serum-free PrEGM. Cells were treated with 2 μmol/L lycopene or placebo beadlets for 48 hours after reaching 70% confluence. Lycopene beadlets have been shown to be stable in cell culture medium for up to 72 hours (20).

Subcellular fractionation

After incubation with lycopene or placebo, the cell culture medium was removed, and cells were washed twice with ice-cold PBS buffer. Hypotonic buffer (200 μL) containing 20 mmol/L Tris (pH 7.5), 5 mmol/L MgCl₂, 5 mmol/L CaCl₂, 1 mmol/L dithiothreitol (DTT), 1 mmol/L EDTA, and 1% protease inhibitor cocktail were added to the cells which were scraped into buffer and transferred to a new tube. Cells were disrupted by repeated freezing and thawing. The crude nuclear pellet was collected by centrifugation at 1,800 × g for 15 minutes at 4°C. The supernatant containing the cytosolic and membrane proteins were frozen at –80°C

until use. The crude nuclear pellet was resuspended on ice in 0.5 volumes of low salt buffer containing 20 mmol/L Tris (pH 7.5), 5 mmol/L MgCl₂, 20 mmol/L KCl, 1 mmol/L DTT, 1 mmol/L EDTA, and 1% protease inhibitor cocktail. While the nuclei were on ice, 0.5 nuclear volumes of high salt buffer containing 20 mmol/L Tris (pH 7.5), 5 mmol/L MgCl₂, 1.2 mol/L KCl, 1 mmol/L DTT, 1 mmol/L EDTA, and 1% protease inhibitor cocktail were added slowly to solubilize nuclear proteins. Triton-X100 (1%) was added to the suspension that was sonicated 4 times and centrifuged at 25,000 × g for 30 minutes at 4°C to pellet nuclear debris. The supernatant, which contained nuclear and nuclear membrane proteins, was stored at –80°C until use. The protein concentration of each cell fraction was determined by using the Bio-Rad protein assay according to manufacturer's instructions.

Protein labeling by iTRAQ

Proteins from each fraction were digested by using trypsin and labeled with iTRAQ reagents following the manufacturer's protocol with some modification. Briefly, 100 μg protein from each fraction was precipitated by acetone at –20°C for 2 hours. Each protein pellet was dissolved in 0.5 mol/L triethylammonium bicarbonate buffer with 0.1% sodium dodecylsulfate and reduced in 5 mmol/L tris(2-carboxyethyl)phosphine at 60°C for 1 hour. The reduced protein was blocked in 10 mmol/L methyl methanethiosulfonate by incubating at room temperature for 20 minutes and then digested at 37°C overnight by trypsin (Promega) with shaking. iTRAQ reagent in ethanol was added to each sample (>60% ethanol in the reaction), and the reaction mixture was incubated at room temperature for 2 hours. The reaction was stopped by adding an equal volume of water, and the experiment and control samples were mixed together for mass spectrometric analysis.

Two-dimensional microcapillary HPLC-tandem mass spectrometry

A PolySulfoethyl A SCX column (5 μm, 200 Å, 4.6 × 100 mm) from PolyLC was used to fractionate digested iTRAQ-labeled peptides before reversed-phase two-dimensional microcapillary HPLC-tandem mass spectrometry (μLC-MS/MS). Mobile phase A consisted of 10 mmol/L potassium phosphate (pH < 3) and 25% acetonitrile, and mobile phase B consisted of 10 mmol/L potassium phosphate (pH < 3), 1 mol/L KCl, and 25% acetonitrile. Labeled peptides were diluted with 25% acetonitrile in water (pH < 3) at least 10-fold to reduce the concentration of buffer and iTRAQ reagents, loaded onto the SCX column, and eluted as follows: 100% mobile phase A for 5 minutes, 0% to 10% mobile phase B over 5 minutes, 10% to 25% mobile phase B over 25 minutes, 25% to 50% mobile phase B over 10 minutes, 50% B for 5 minutes, and then 100% mobile phase A for 20 minutes. Fractions were collected each minute and combined according to UV 280 nm absorbance. The fractions were evaporated to dryness under a stream of nitrogen and reconstituted in 4% acetonitrile in water containing 0.1% formic acid immediately before μLC-MS/MS analysis.

Labeled peptides were analyzed using a Thermo LTQ linear ion trap mass spectrometer equipped with a Dionex μ HPLC system. Reverse-phase μ HPLC was carried out using an Agilent Zorbax 300SB C_{18} column (3.5 μ m, 75 μ m \times 150 mm) and Dionex/LC Packings C_{18} PepMap precolumn cartridge (5 μ m, 0.3 mm \times 5 mm). A linear gradient was used from 5% to 55% solvent B over 120 minutes (solvent A: 95:5:0.1 and solvent B: 5:95:0.1, water/acetonitrile/formic acid, v/v/v) at a flow rate of 250 nL/minute. Positive ion nano-electrospray mass spectra were acquired in data-dependent mode in which each mass spectrometry scan (m/z 400–2,000) was followed by 4 tandem mass spectrometry (MS/MS) scans using hybridization of pulsed Q dissociation (PQD) with a normalized collision energy of 31%, activation Q value of 0.6, activation time of 0.4 ms, and collision-induced dissociation (CID) with collision energy of 35%, activation Q of 0.25, and activation time of 30 ms. The 4 most abundant peptide ions in each mass spectrum except singly charged ions were dynamically selected to generate tandem mass spectra. Dynamic exclusion was used to prevent repetitive selection of the same ions during the next 60 seconds.

The iTRAQ experiments were carried out with 3 independent biologic replicates. Each of the 3 biologic experiments was analyzed 3 times by μ LC-MS/MS as technical replicates to gather reliable quantitative information. Collision-induced dissociation and PQD were used to obtain reliable quantitation and sequencing of peptide ions.

Western blot analysis

PrE cells were used on the second passage at 70% confluency. Lycopene or placebo beadlets were dissolved in cell culture media at a final concentration of 2 μ mol/L (or equivalent mass of placebo), and cell media were replaced with the test containing media for 48 hours. Protein samples were collected in Cell Lysis Buffer (Cell Signaling Technology). After a 10-minute centrifugation at 10,000 \times g and 4°C, clarified protein lysate (10 μ g) was run on a 10% bis-Tris NuPAGE gel (Life Technologies) and transferred to a polyvinylidene difluoride membrane. Membranes were blocked in either 5% milk or bovine serum albumin depending on the antibody manufacturer's recommendations and incubated with antibodies at the following concentrations: HSP90 β 1 (Grp94) 1:1,000; NDRG1 1:5,000; GSTP1 1:5,000, and Rad23b (Aka hHR23b) 1:500. Anti-mouse-horseradish peroxidase (HRP) and anti-rabbit-HRP (Cell Signaling) were used at 1:5,000 and the bands visualized with Western Lightning ECL Pro (PerkinElmer). Densitometry measurements of the band intensities were carried out based on mean peak height with ImageJ 1.46r (NIH, Bethesda, MD).

Data analysis

The raw data from the MS/MS analyses were extracted automatically and converted to mzXML and MGF formats by using in house software. Two different search engines, MassMatrix (University of Illinois at Chicago, IL; refs. 21, 22) and Mascot (Matrix Science) were used to search against

the International Protein Index (IPI.human.V3.5, 2008) with decoy option to improve confidence levels of protein quantitation and identification. The mass tolerance for precursor ions and fragment ions was set to 2 and 0.8 Da, respectively. Up to 2 missed trypsin cleavages were allowed. Variable modification was permitted to allow for the detection of methionine oxidation, and fixed modifications included β -methylthiolation of cysteine and the iTRAQ MS/MS tag of peptide N-terminals and lysine. Mascot searching was used to identify the protein hits, and the Mascot search results were further visualized and validated by Scaffold (Proteome Software). MassMatrix was used to quantify the relative changes in protein expression and the individual protein expression ratio change was normalized by protein global expression ratio as described by Armenta and colleagues (23). For chemoprevention, a 10% change in protein expression was defined as significant.

Results and Discussion

Proteomics of human primary prostatic epithelial cells

A model of transit amplifying cells of the basal prostatic epithelium, PrE cells have a basal phenotype as evidenced by p63 and CK5 positivity and lack of an androgen receptor as determined using quantitative real-time PCR and have a high rate of proliferation (17, 24). They express basal and transitional cell type markers (25) and cytokeratins 5 and 14, which were confirmed during proteomics measurements (Supplementary Table S1). These basal cells are believed to be the cell origin for common prostatic adenocarcinomas, which has been confirmed by Goldstein and colleagues (18) using human primary prostate basal cells with lentivirus carrying genetic alteration genes in immunodeficient mice.

In the cytosolic and membrane fraction of the PrE cells, 371 proteins were identified including 254 proteins shared by all 3 experiments, 93 proteins identified in 2 experiments, and 24 proteins identified in just a single experiment with a false discovery rate of 0.0% protein and peptides (Fig. 1). All the proteins identified have a probability score of at least 95% ($P \leq 0.05$) with at least 2 unique peptides, and the peptide probability score was 90% or more. Gene ontology analysis of biologic processes of the identified proteins in the cytosolic and membrane fractions indicated that most of these proteins are involved in metabolism, development, response to stimulus, and regulatory processes; the remainder of the identified proteins are involved in immune, growth, multicellular, and multiorganism processes. Gene ontology analysis of molecular function indicated that most of the identified proteins have binding and catalytic activities, and that the other proteins have primarily antioxidant, transporter, translation regulator, enzyme regulator, carrier, and motor activities. Therefore, many proteins that were identified in the cytosolic and membrane fraction might be involved in mechanisms of action related to lycopene.

In the nuclear extract fraction, 266 proteins were identified including 159 proteins by all 3 experiments, 76 proteins

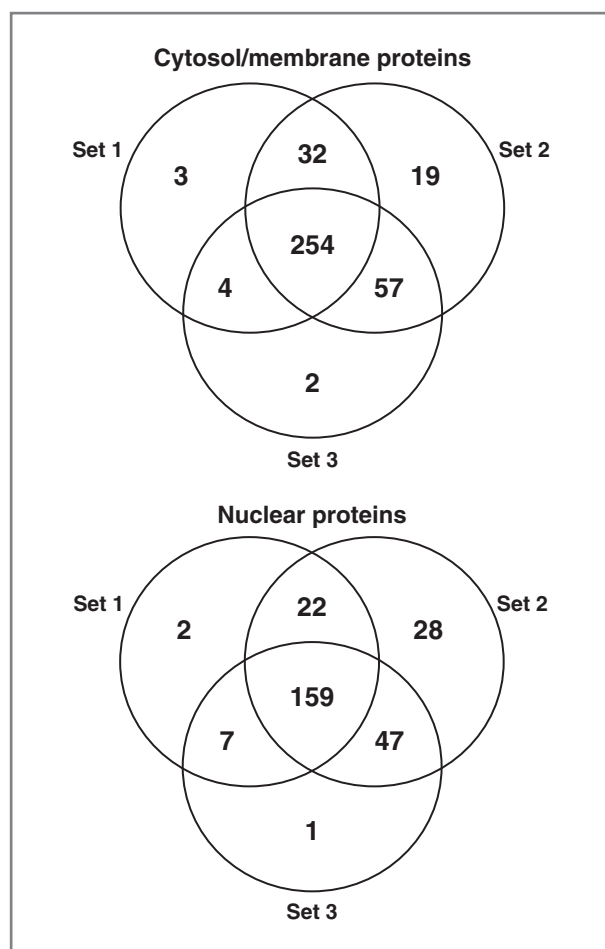


Figure 1. Venn diagrams of proteins identified during 3 independent biologic experiments in the cytosol/membrane fraction and in the nuclear fraction of human primary PrE cells treated with lycopene.

in 2 experiments, and 31 proteins identified in just one experiment. The false discovery rate for protein and peptides was 0.8% and 0.1%, respectively. The gene ontology analysis of biologic processes of the proteins identified in nuclear extracts indicated that most of the identified proteins were involved in metabolism, development, cell proliferation, cell growth, and regulatory processes. The other nuclear proteins that were identified were involved in communication, cell killing, immune response, and cell aging processes. Gene ontology analysis of molecular function of the nuclear proteins indicated that most of the identified proteins have binding, catalytic, and structural activities, followed by transporter, translation, transcription, and antioxidant functions.

Differential protein expression induced by lycopene

The cytosolic membrane fraction and nuclear extract protein expression level changes showed an average change close to 1.0, and this distribution met normalization requirements based on protein global expression ratio (23). The proteins modulated by lycopene treatment com-

pared with placebo were identified and are listed in Table 1 according to their cellular functions which included modulating cell redox homeostasis, apoptosis, cytoprotection, and androgen signaling. Note that these proteins showed the same expression change in at least 2 independent experiments.

A representative positive ion electrospray PQD and CID tandem mass spectrum of a peptide from glutathione S-transferase-pi-1 (GSTP1) is shown in Fig. 2. The amino acid sequence of this peptide was determined on the basis of the indicated *b* ions and γ ions. Figure 2B shows the low mass region containing the iTRAQ ion of *m/z* 145 and the iTRAQ reporter ions of *m/z* 114 and *m/z* 116 from which the relative amounts of the peptides were determined.

Apoptosis

Lycopene treatment of the PrE cells upregulated proteins associated with apoptosis induction and downregulated proteins involved in antiapoptotic processes (Table 1). The proteins associated with apoptosis induction that were upregulated were tyrosyl-tRNA synthetase (TyrRS; upregulated by 20%), 40S ribosomal protein S3 (RPS3; up by 15%), and pyruvate kinase isozyme M2 (PKM2; up by 15%). Wakasugi and colleagues (26) reported that upregulation of TyrRS is associated with cellular apoptosis. Upregulation of RPS3 can result in apoptosis through activation of caspase, whereas upregulation of PKM2 is important for caspase-independent cell death of tumor cells.

The apoptotic proteins downregulated by lycopene treatment (Table 1) included chloride intracellular channel protein 1 (CLIC1; downregulated by 35%), heat shock 70 kDa protein (HSP70) 1A/1B (down by 12%), HSP β 1 (HSP27; down by 10%), Rho GDP-dissociation inhibitor 1 (Rho GDI 1; down by 11%), translationally controlled tumor protein (TCTP; down by 10%), lactoylglutathione lyase (down by 13%), 78 kDa glucose-regulated protein (Grp78; down by 16%), and protein kinase C inhibitor protein 1 (KCIP1; down by 15%). Suppression of CLIC1 protein can induce apoptosis, enhance TNF- α -induced apoptosis, and inhibit tumor growth as reported by Suh and colleagues (27). HSP70 directly inhibits apoptosis by blocking the recruitment of procaspase-9 to the Apaf-1/dATP/cytochrome c apoptosome complex (28). HSP27 protects cancer cells against apoptosis by interacting with the outer mitochondrial membranes and interfering with the activation of cytochrome/c/Apaf-1/dATP complex to inhibit the activation of procaspase-9 (29). Zhang and colleagues (30) reported that Rho GDI 1 protects cancer cells against drug-induced apoptosis. TCTP is stabilized by antiapoptotic protein myeloid cell leukemia sequence (31), and its level is downregulated through activation of the tumor suppressor protein p53 (32). Grp78 expression is associated with the development of castration-resistant prostate cancer (33). KCIP1 is known to bind to MEKK1, which is activated by caspase 3 to block apoptosis (34).

The apoptotic activity of lycopene is consistent with the results of the study conducted by Ivanov and colleagues (35) and Hwang and colleagues (36) who reported

Table 1. Human primary prostate epithelial cellular proteins differentially expressed due to lycopene treatment

Accession number	Protein	Unique peptides	Fold change ($P \leq 0.05$)	Function
Cytosol/membrane (nonnuclear)				
Apoptosis				
IP100021263	Protein Kinase C inhibitor protein 1	7	0.85 ± 0.13	Antiapoptosis process
IP100304925	Heat shock 70 kDa protein 1A/1B	6	0.88 ± 0.10	Antiapoptosis process
IP100025512	HSPβ-1	4	0.90 ± 0.04	Antiapoptosis process
IP100003815	Rho GDP-dissociation inhibitor 1	3	0.89 ± 0.09	Antiapoptosis process
IP100550900	Translationally controlled tumor protein	2	0.90 ± 0.07	Antiapoptosis process
IP100220766	Lactylglutathione lyase	4	0.87 ± 0.01	Antiapoptosis process
IP100003362	78 kDa glucose-regulated protein	14	0.84 ± 0.09	Antiapoptosis process
IP100007074	Tyrosyl-tRNA synthetase	3	1.20 ± 0.09	Induction of apoptosis
IP100011253	40S ribosomal protein S3	6	1.15 ± 0.07	Activation of caspase and induction of apoptosis
IP100479186	Pyruvate kinase isozymes M2	19	1.15 ± 0.07	Nuclear translocation induction of cell death
IP100010896	Chloride intracellular channel protein 1	6	0.65 ± 0.05	Suppression of CLIC1 which induces apoptosis and inhibits tumor growth
Antioxidation and cytoprotection				
IP100386755	ERO1-like protein alpha	10	0.80 ± 0.06	Responsible for a significant proportion of ROS
IP100642936	Glutathione S-transferase omega 1	4	1.11 ± 0.05	Functions in the GSH-ascorbate cycle as part of antioxidant metabolism
IP100009634	Sulfide-quinone oxidoreductase	8	1.13 ± 0.06	Catalyzes oxidation of hydrogen sulfide with the help of a quinone
IP100640741	Peroxiredoxin-1	7	1.14 ± 0.09	Reduces hydrogen peroxide and alkyl hydroperoxides
IP100219757	Glutathione S-transferase P 1	10	1.17 ± 0.04	Conjugates GSH with electrophiles
IP100000877	Hypoxia upregulated protein 1	4	1.56 ± 0.19	Cytoprotective mechanisms triggered by oxygen deprivation
IP100008223	UV excision repair protein RAD23 homolog B	3	1.49 ± 0.35	Recognizes helix-distorting lesions in DNA and initiates global genome repair
Signal transduction				
IP100298547	Protein DJ1	4	0.77 ± 0.13	Positive regulator of androgen receptor-dependent transcription
IP100027230	HSP90β1	9	0.89 ± 0.08	AR chaperone protein which stabilizes AR in the cytosol
IP100646689	Thioredoxin domain-containing protein 17	3	0.87 ± 0.07	Deficiency enhances TNF-α-induced apoptosis
IP100293276	Macrophage migration inhibitory factor	2	0.76 ± 0.04	Proinflammatory cytokine and positive regulation of ERK1 and ERK2 cascade
IP100022078	Protein NDRG1	4	1.42 ± 0.21	Act as tumor suppressors in many cell types
IP100411765	Epithelial cell marker protein 1	7	0.88 ± 0.08	Regulates protein synthesis and epithelial cell growth by stimulating Akt/mTOR pathway
Nucleus				
IP100021405	Prelamin-A/C	7	0.84 ± 0.14	Disrupts mitosis and induces DNA damage
IP100411765	Epithelial cell marker protein 1	7	0.84 ± 0.08	Regulates protein synthesis and epithelial cell growth by stimulating Akt/mTOR pathway

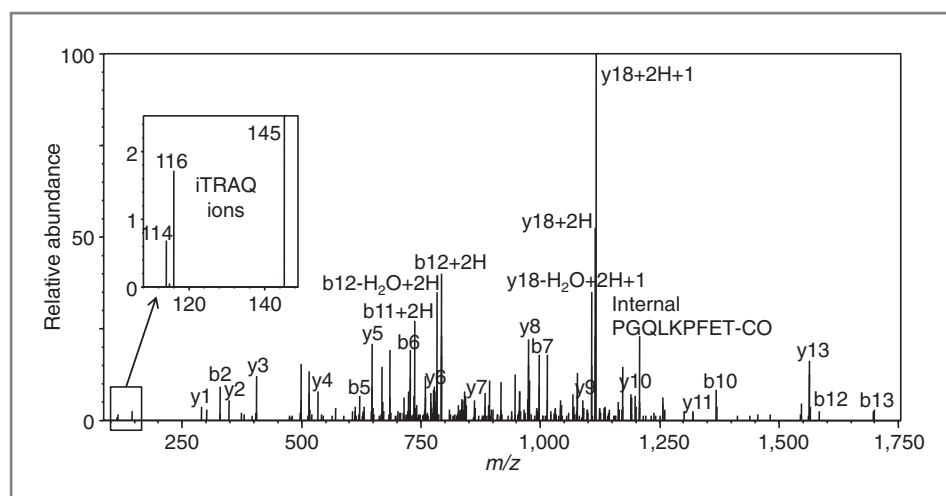


Figure 2. Positive ion nano-electrospray P/QD and collision-induced dissociation product ion tandem mass spectrum of peptide ALPGQLKPFETLLSQNQGGK from GSTP1 protein which was upregulated in the cytosolic/membrane fraction. The triply protonated precursor ion was observed at m/z 853.831 corresponding to an uncharged mass of 2558.47 u (ΔM 2.2 ppm). Note the iTRAQ reporter ions of m/z 114, m/z 116 and m/z 145 that were used for the determination of relative protein expression in PrE cells treated with lycopene or placebo beadslets.

apoptosis induction in prostate cancer cells by lycopene and Bowen and colleagues (37) and Kim and colleagues (38) who found that lycopene increased the apoptotic index in neoplastic and hyperplastic human prostate tissues. These results indicate that lycopene exerts proapoptotic effects in PrE cells by downregulating expression of antiapoptotic chaperone proteins, activating caspase activity, and promoting the caspase-independent cell death pathway. However, treatment of PrE cell cultures with lycopene for 48 hours did not produce any observable apoptosis.

Cytoprotection and redox homeostasis

GSTP1, which catalyzes the conjugation of exogenous and endogenous electrophilic compounds with glutathione, was upregulated 17% in PrE cells treated with lycopene (Table 1). Silencing of the gene for GSTP1 by promoter hypermethylation occurs frequently in prostate cancer and has been detected in proliferative inflammatory atrophy lesions of the prostate (39, 40). Glutathione-S-transferase omega 1 (GSTO1), which functions in the glutathione-ascorbate cycle to detoxify hydrogen peroxide, was upregulated by 11%. Peroxiredoxin 1, an antioxidant enzyme that reduces hydrogen peroxide and alkyl hydroperoxides, was upregulated by 14%, and sulfide-quinone oxidoreductase, which catalyzes the oxidation of hydrogen sulfide, was upregulated by 13% in PrE cells treated with lycopene (Table 1). These effects indicate that another function of lycopene is to increase levels of phase II protective enzymes that can prevent cytotoxicity due to xenobiotic electrophiles and carcinogens.

These results are consistent with those of Ben-Dor and colleagues (10) who reported that lycopene induced the antioxidant response element in MCF-7 human mammary cancer cells and in HepG2 human hepatocellular carcinoma cells. Our results show that lycopene upregulates phase II enzymes in PrE cells and not just in mammary or liver cells, and our data show a chemoprevention effect in the primary prostate epithelial cells instead of cancer cells. Establishing upregulation of phase II enzymes after lycopene exposure is

significant in terms of confirming that this prostate cancer chemoprevention mechanism occurs in human PrE cells.

Hypoxia upregulated protein 1, which participates in cytoprotective cellular mechanisms triggered by oxygen deprivation, was upregulated by 56% in PrE cells treated with lycopene (Table 1). The UV excision repair protein RAD23 homolog B, which has a central role in DNA repair, was upregulated by 49% (Table 1). Both of these effects indicate that lycopene stimulates cytoprotective functions in PrE cells that can help prevent prostate cancer initiation.

Lycopene treatment of PrE cells downregulated ERO1-like protein alpha by 20% (Table 1). ERO1-like protein alpha is a cytosolic protein responsible for a significant proportion of reactive oxygen species (ROS) in cells. CLIC1, which is usually expressed during oxidative stress, was downregulated by 35% (Table 1). These results show that lycopene can reduce ROS generation and reduce oxidative stress in PrE cells. These results are consistent with reports that lycopene reduces oxidative DNA damage in CV1-P monkey cell culture (41), in rat prostate tissue (42), and in human prostate tissue (43).

Signal transduction interference

Lycopene has been reported to alter signaling in the prostate including inflammation signaling, growth factor signaling, and steroid hormone signaling (44). Lycopene treatment of PrE cells downregulated thioredoxin domain-containing protein 17 (TXNDC17) by 13% (Table 1). Deficiency of TXNDC17 enhances TNF- α -induced activation of caspase and subsequent apoptosis (45). Macrophage migration inhibitory factor, which is a proinflammatory cytokine and a positive regulator of the mitogen-activated protein kinase (MAPK) pathway, was downregulated by 24% (Table 1). This effect can reduce inflammation, inhibit cell proliferation, and slow prostate cancer progression to androgen-independent growth (4, 46).

Protein DJ1, a positive regulator of androgen receptor-dependent transcription, was downregulated by 23% in PrE cells (Table 1), indicating that lycopene exerts antiandrogen

activity at the level of gene expression. HSP90, a chaperone protein that helps to stabilize the androgen receptor in the cytosol, was downregulated by 11% (Table 1). Inhibitors of HSP90 cause androgen receptor degradation and are in clinical trials for prostate cancer therapy (47). These results are consistent with those of Herzog and colleagues (48) and Siler and colleagues (15) who showed that lycopene reduced androgen signaling and lowered expression of androgen receptor target genes including prostatic steroid binding protein C1 and C3, cystatin-related protein 2, and seminal vesicle secretion protein IV. Downregulation of androgen signaling by lycopene is an anticancer effect that should decrease androgen-dependent prostate growth.

Protein NDGR1, which is a tumor suppressor in many cell types and is necessary for the p53/TP53-mediated caspase activation and apoptosis, was upregulated by 42% in PrE cells treated with lycopene (Table 1). Epithelial cell marker protein 1 (SFN), which regulates protein synthesis and epithelial cell growth by stimulating the AKT/mTOR pathway, was downregulated by 12% (Table 1). Estimated to be upregulated 30% to 50% in most prostate cancers (2), the PI3K/AKT/mTOR pathway is a target for prostate cancer therapy. Reduced AKT signaling by lycopene might prevent the progression of prostate cancer to an androgen-independent state. These observations are consistent with lycopene growth inhibitory effects reported by other groups studying prostate cancer cells and PrE cells (35, 36, 49). Repression of the AKT/mTOR pathway by downregulation of SFN would contribute to chemoprevention by lycopene by inhibiting prostate cancer progression to an androgen-independent state.

Compared with cytosolic and membrane proteins, the expression of relatively few nuclear proteins in PrE cells was found to be altered by lycopene. Downregulated by 12% in the cytosol, the expression of SFN in the nucleus was reduced 16% in response to lycopene treatment (Table 1). Finally, lycopene downregulated prelamin-A/C 16% in the nucleus (Table 1). Prelamin-A/C disrupts mitosis and induces DNA damage.

To help confirm the proteomics results, identification and differential expression of 4 proteins in cell cultures established from 3 different patients were investigated using Western blot analysis. The presence of RAD23B, NDRG1, GSTP1, and HSP90 β 1 was confirmed in all 3 cell lines (Supplementary Fig. S1), and differential expression of each protein due to lycopene treatment was similar to the proteomics results. For example, HSP90 β 1 was downregulated in 2 of 3 cell lines, and RAD23B, NDRG1, and GSTP1 were upregulated in 2 of 3 cell lines.

References

1. National Cancer Institute, Surveillance Epidemiology and End Results 2012; [Accessed 2012 Aug 10]. Available from: <http://seer.cancer.gov/statfacts/html/prost.html>.

Conclusions

Prostate cancer initiation, promotion, and progression involve multiple molecular pathways, many of which interact with each other. Investigations of changes in the levels of one or just a few proteins due to lycopene exposure cannot detect such pleiotropic effects. Our proteomics approach shows how lycopene exerts multiple protective effects on human PrE cells while avoiding the use of less relevant models of chemoprevention such as prostate cancer cells or even nonprostate cell lines.

Several proteins that were upregulated or downregulated by lycopene indicate reduced oxidative stress in the cell. Upregulation of phase II enzymes such as GSTP1, GSTO1, and SQR can help prevent cancer initiation by detoxifying potentially carcinogenic electrophiles. Lycopene was found to inhibit proliferation of PrE cells by downregulating the AKT/mTOR pathway and by upregulating genes that have growth inhibitory effects. Lycopene upregulated proteins that can promote apoptosis and downregulate several proteins involved in antiapoptosis. Lycopene was also found to alter several signaling pathways, including inhibition of androgen signaling, downregulation of TNF- α signaling, and deactivation of the MAPK pathway. All of these lycopene effects on cellular proteins contribute to the prevention of cancer initiation, promotion, and progression and are consistent with many previous studies that have addressed just one or a few of these effects of lycopene.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: X. Qiu, L. Nonn, R.B. van Breemen

Development of methodology: X. Qiu, Y. Yuan, A. Vaishnav, L. Nonn, R.B. van Breemen

Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): X. Qiu, Y. Yuan, L. Nonn, M.A. Tessel, R.B. van Breemen

Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): X. Qiu, Y. Yuan, L. Nonn, M.A. Tessel, R.B. van Breemen

Writing, review, and/or revision of the manuscript: X. Qiu, Y. Yuan, L. Nonn, R.B. van Breemen

Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): R.B. van Breemen

Study supervision: R.B. van Breemen

Grant Support

This research was supported by the National Cancer Institute (grant R01 CA101052; to R.B. van Breemen).

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Received August 23, 2012; revised January 31, 2013; accepted February 8, 2013; published OnlineFirst March 12, 2013.

2. Morgan TM, Koreckij TD, Corey E. Targeted therapy for advanced prostate cancer: inhibition of the PI3K/Akt/mTOR pathway. *Curr Cancer Drug Targets* 2009;9:237-49.

3. van Breemen RB, Sharifi R, Viana M, Pajkovic N, Zhu D, Yuan L, et al. Antioxidant effects of lycopene in African American men with prostate cancer or benign prostate hyperplasia: a randomized, controlled trial. *Cancer Prev Res* 2011;4:711–8.
4. Di Mascio P, Kaiser S, Sies H. Lycopene as the most efficient biological carotenoid singlet oxygen quencher. *Arch Biochem Biophys* 1989; 274:532–8.
5. Riso P, Visioli F, Grande S, Guarnieri S, Gardana C, Simonetti P, et al. Effect of a tomato-based drink on markers of inflammation, immunomodulation, and oxidative stress. *J Agric Food Chem* 2006;54:2563–6.
6. Haseen F, Cantwell MM, O'Sullivan JM, Murray LJ. Is there a benefit from lycopene supplementation in men with prostate cancer? A systematic review. *Prostate Cancer Prostatic Dis* 2009;12:325–32.
7. Hazai E, Bikadi Z, Zsila S, Lockwood SF. Molecular modeling of the non-covalent binding of the dietary tomato carotenoids lycopene and lycophyl, and selected oxidative metabolites with 5-lipoxygenase. *Biorg Med Chem* 2006;14:6859–67.
8. Karas M, Amir H, Fishman D, Danilenko M, Segal S, Nahum A, et al. Lycopene interferes with cell cycle progression and insulin-like growth factor I signaling in mammary cancer cells. *Nutr Cancer* 2000;36: 101–11.
9. Chew BP, Park JS. Carotenoid action on the immune response. *J Nutr* 2004;134:257S–261S.
10. Ben-Dor A, Steiner M, Gheber L, Danilenko M, Dubi N, Linnewiel K, et al. Carotenoids activate the antioxidant response element transcription system. *Mol Cancer Ther* 2005;4:177–86.
11. Levy J, Bosin E, Feldman B, Giata Y, Miinster A, Danilenko M, et al. Lycopene is a more potent inhibitor of human cancer cell proliferation than either alpha-carotene or beta-carotene. *Nutr Cancer* 1995;24: 257–66.
12. Rao AV, Fleshner N, Agarwal S. Serum and tissue lycopene and biomarkers of oxidation in prostate cancer patients: a case-control study. *Nutr Cancer* 1999;33:159–64.
13. Zhang L-X, Cooney RV, Bertram JS. Carotenoids enhance gap junctional communication and inhibit lipid peroxidation in C3H/10T1/2 cells: relationship to their cancer chemopreventive action. *Carcinogenesis* 1991;12:2109–14.
14. Palozza P, Parrone N, Simone R, Catalano A. Role of lycopene in the control of ROS-mediated cell growth: implications in cancer prevention. *Curr Med Chem* 2011;18:1846–60.
15. Siler U, Barella L, Spitzer V, Schnorr J, Lein M, Goralczyk R, et al. Lycopene and vitamin E interfere with autocrine/paracrine loops in the Dunning prostate cancer model. *FASEB J* 2004;18:1019–21.
16. Goo YA, Li Z, Pajkovic N, Shaffer S, Taylor G, Chen J, et al. Systematic investigation of lycopene effects in LNCaP cells by use of novel large-scale proteomic analysis software. *Proteomics Clin Appl* 2007;1: 513–23.
17. Uzgaré AR, Xu Y, Isaacs JT. *In vitro* culturing and characteristics of transit amplifying epithelial cells from human prostate tissue. *J Cell Biochem* 2004;91:196–205.
18. Goldstein AS, Huang J, Guo C, Garraway IP, Witte ON. Identification of a cell of origin for human prostate cancer. *Science* 2010;329: 568–71.
19. Peehl DM. Growth of prostatic epithelial and stromal cells *in vitro*. In: Russell PJ, Jackson P, Kingsley EA, editors. *Prostate Cancer Methods and Protocols*. Totowa, NJ: Human Press; 2003. p. 41–57.
20. Liu A, Pajkovic N, Pang Y, Zhu D, Calamini B, Mesecar AL, et al. Absorption and subcellular localization of lycopene in human prostate cancer cells. *Mol Cancer Ther* 2006;5:2879–85.
21. Xu H, Freitas MA. MassMatrix: a database search program for rapid characterization of proteins and peptides from tandem mass spectrometry data. *Proteomics* 2009;9:1548–55.
22. Xu H, Freitas MA. A mass accuracy sensitive probability based scoring algorithm for database searching of tandem mass spectrometry data. *BMC Bioinformatics* 2007;8:133.
23. Armenta JM, Hoeschele I, Lazar IM. Differential protein expression analysis using stable isotope labeling and PQD linear ion trap MS technology. *J Am Soc Mass Spectrom* 2009;20:1287–302.
24. Peehl DM. Are primary cultures realistic models of prostate cancer? *J Cell Biochem* 2004;91:185–95.
25. Peehl DM. Primary cell cultures as models of prostate cancer development. *Endocr Relat Cancer* 2005;12:19–47.
26. Wakasugi K, Schimmel P. Highly differentiated motifs responsible for two cytokine activities of a split human tRNA synthetase. *J Biol Chem* 1999;274:23155–9.
27. Suh KS, Mutoh M, Gerdes M, Crutchley JM, Mutoh T, Edwards LE, et al. Antisense suppression of the chloride intracellular channel family induces apoptosis, enhances tumor necrosis factor α -induced apoptosis, and inhibits tumor growth. *Cancer Res* 2005;65:562–71.
28. Beere HM, Wolf BB, Cain K, Mosser DD, Mahboubi A, Kuwana T, et al. Heat-shock protein 70 inhibits apoptosis by preventing recruitment of procaspase-9 to the Apaf-1 apoptosome. *Nat Cell Biol* 2000;2:469–75.
29. Sarto C, Binz PA, Mocarelli P. Heat shock proteins in human cancer. *Electrophoresis* 2000;21:1218–26.
30. Zhang B, Zhang Y, Dagher MC, Shacter E. Rho GDP dissociation inhibitor protects cancer cells against drug-induced apoptosis. *Cancer Res* 2005;65:6054–62.
31. Zhang D, Li F, Weidner D, Mnjoyan ZH, Fujise K. Physical and functional interaction between myeloid cell leukemia 1 protein (MCL1) and fortilin. *J Biol Chem* 2002;277:37430–8.
32. Tuynder M, Susini L, Prieur S, Besse S, Fiucci G, Amson R, et al. Biological models and genes of tumor reversion: cellular reprogramming through tpt1/TCTP and SIAH-1. *Sci STKE* 2002;99:14976–81.
33. Pootrakul L, Datar RH, Shi SR, Cai J, Hawes D, Groshen SG, et al. Expression of stress response protein Grp78 is associated with the development of castration-resistant prostate cancer. *Clin Cancer Res* 2006;12:5987–93.
34. Fanger GR, Widmann C, Porter AC, Sather S, Johnson GL, Vaillancourt RR. 14-3-3 proteins interact with specific MEK kinases. *J Biol Chem* 1998;273:3476–83.
35. Ivanov NI, Cowell SP, Brown P, Rennie PS, Guns ES, Cox ME. Lycopene differentially induces quiescence and apoptosis in androgen-responsive and -independent prostate cancer cell lines. *Clin Nutr* 2007;26:252–63.
36. Hwang ES, Bowen PE. Cell cycle arrest and induction of apoptosis by lycopene in LNCaP human prostate cancer cells. *J Med Food* 2004;7:284–9.
37. Bowen P, Chen L, Stacewicz-Sapuntzakis M, Duncan C, Sharifi R, Ghosh L, et al. Tomato sauce supplementation and prostate cancer: lycopene accumulation and modulation of biomarkers of carcinogenesis. *Exp Biol Med* 2002;227:886–93.
38. Kim HS, Bowen P, Chen L, Duncan C, Ghosh L, Sharifi R, et al. Effects of tomato sauce consumption on apoptotic cell death in prostate benign hyperplasia and carcinoma. *Nutr Cancer* 2003;47:40–7.
39. Nakayama M, Bennett CJ, Hicks JL, Epstein JI, Platz EA, Nelson WG, et al. Hypermethylation of the human glutathione S-transferase-pi gene (GSTP1) CpG island is present in a subset of proliferative inflammatory atrophy lesions but not in normal or hyperplastic epithelium of the prostate: a detailed study using laser-capture microdissection. *Am J Pathol* 2003;163:923–33.
40. Lee WH, Isaacs WB, Bova GS, Nelson WG. CG island methylation changes near the GSTP1 gene in prostatic carcinoma cells detected using the polymerase chain reaction: a new prostate cancer biomarker. *Cancer Epidemiol Biomarkers Prev* 1997;6:443–50.
41. Matos HR, Di Mascio P, Medeiros MH. Protective effect of lycopene on lipid peroxidation and oxidative DNA damage in cell culture. *Arch Biochem Biophys* 2000;383:56–9.
42. Matos HR, Marques SA, Gomes OF, Silva AA, Heimann JC, Di Mascio P, et al. Lycopene and beta-carotene protect *in vivo* iron-induced oxidative stress damage in rat prostate. *Braz J Med Biol Res* 2006;39:203–10.
43. Chen L, Stacewicz-Sapuntzakis M, Duncan C, Sharifi R, Ghosh L, van Breemen RB, et al. Oxidative DNA damage in prostate cancer patients consuming tomato sauce-based entrees as a whole-food intervention. *J Natl Cancer Inst* 2001;93:1872–9.
44. Wertz K. Lycopene effects contributing to prostate health. *Nutr Cancer* 2009;61:775–83.
45. Jeong W, Chang TS, Boja ES, Fales HM, Rhee SG. Roles of TRP14, a thioredoxin-related protein in tumor necrosis factor- α signaling pathways. *Sci STKE* 2004;279:3151–9.

46. Kim GY, Kim JH, Ahn SC, Lee HJ, Moon DO, Lee CM, et al. Lycopene suppresses the lipopolysaccharide-induced phenotypic and functional maturation of murine dendritic cells through inhibition of mitogen-activated protein kinases and nuclear factor-kappaB. *Immunology* 2004;113:203–11.
47. Taplin ME. Drug insight: role of the androgen receptor in the development and progression of prostate cancer. *Nat Clin Pract Oncol* 2007;4:236–44.
48. Herzog A, Siler U, Spitzer V, Seifert N, Denelavas A, Hunziker PB, et al. Lycopene reduced gene expression of steroid targets and inflammatory markers in normal rat prostate. *FASEB J* 2005;19:272–4.
49. Obermuller-Jevic UC, Olano-Martin E, Corbacho AM, Eiserich JP, van der Vliet A, Valacchi G, et al. Lycopene inhibits the growth of normal human prostate epithelial cells *in vitro*. *J Nutr* 2003;133:3356–60.