DIETARY LUTEIN MODULATES EXPRESSION OF PROSTATE CANCER BIOMARKER GENES IN HUMAN PROSTATE CANCER CELL LINE

by

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ABSTRACT OF THE THESIS

Dietary Lutein Modulates Expression of Prostate Biomarker Genes in Human Prostate Cancer Cell Line

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Prostate cancer is the second leading cause of death from malignancies in men and is the most commonly diagnosed cancer in the United States. Epidemiological studies have shown the inverse relationship between consumption of various carotenoids and the risk of prostate cancer. Lutein is a fat-soluble, oxycarotenoid present in human serum and is also present in the liver, colon, lung and prostate tissues. Lutein is not synthesized by the human body and is primarily consumed from dark-green leafy vegetables such as kale and spinach, as well as from egg yolks, avocado, corn and fruits like orange and kiwi. Lutein has gained popularity through its role in preventing age-related macular degeneration (AMD). Anti-inflammatory activity of lutein has also been the focus of a number of *in vitro* and *in vivo* studies. Recently much attention has focused on the role of lutein against various cancers including prostate cancer, however no mechanism of action was determined.

Our objective is to determine whether lutein modulates prostate cancer biomarker genes in hormone refractory prostate cancer (PC-3) cell lines using Oligo GEArray® DNA Microarray, which contains 263 genes involved in the progression and diagnosis of prostate cancer. PC-3 cells were treated with 10 μM non-toxic concentrations of lutein as determined by MTT cell

viability assay. Isolated RNA was reverse-transcribed to cDNA, transcribed to cRNA and hybridized with microarrays. Microarray results demonstrated the down-regulation of epidermal growth factor receptor (EGFR), insulin-like growth factor 1 receptor (IGF1R), breast cancer gene 1 (BRCA1), cyclin dependant kinase 5 (CDK5), kallikrein 14 (KLK14) and prostate cancer antigen 3 (PCA3). Microarray results also showed the up-regulation of ras association domain family member 1 (RASSF1) and glutathione S-transferase pi 1 (GSTP1). Modulated genes were validated by Real-Time PCR and demonstrated down-regulation of IGF1R, EGFR, BRCA1, CDK5, KLK14 and PCA3 by 83%, 60%, 50%, 44%, 41% and 40% respectively. Similarly, up-regulated genes were also validated by Real-Time PCR and results showed GSTP1 and RASSF1 up-regulated by 82% and 70%. Modulated genes were further analyzed at the translational level using Western Blot. Among all the prostate cancer biomarker genes, IGF1R, EGFR and GSTP1 were most significantly modulated in Real-Time PCR analysis. Western blot analysis demonstrated that lutein treatment down-regulated the protein expression of IGF1R and EGFR by 40.4% and 33.1% while up-regulating GSTP1 by 30.0%. These results demonstrate the potential of lutein to modulate a number of key biomarker genes involved in human prostate cancer proliferation, differentiation, angiogenesis and apoptosis.

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I cannot end without thanking my loving family, on whose enthusiasm and motivation I began my pursuance of a Masters Degree. I have relied on them for guidance and strength throughout my academic career and I am grateful for their support. I dedicate this work to them.

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Chapter I

Introduction

According to the American Cancer Society, prostate cancer will account for approximately 10% of cancer related deaths in men in 2008, preceded solely by lung cancer. Statistics also show an estimated 186,320 new cases and 28,660 deaths caused by prostate cancer alone (American Cancer Society, 2008). Although there are no definite causes of prostate cancer, risk factors include tobacco use, carcinogens, radiation, poor nutritional status and diet, hormones, immune conditions, genetic factors and age (American Cancer Society, 2008). Age is considered the strongest risk factor with 2 out of 3 prostate cancer incidences found in men over the age of 65 (American Cancer Society, 2008). Prostate cancer is also more prevalent in African American men than Caucasian and Hispanic men, with significantly fewer cases in first generation Asian American men. Family history and poor diet have also been implicated as factors for prostate cancer risk (Clinton and Giovannucci 1998; American Cancer Society, 2008).

The prostate, an accessory gland, is part of the male reproductive system and is located in front of the rectum, below the bladder and wraps around the urethra. Its main role is to aid in sperm function by producing seminal fluid (Clinton and Giovannucci 1998). The proximity of the prostate to the bladder and urethra explains the most common symptoms of prostate cancer including an inability to urinate, weak urine flow, painful urination, presence of blood in urine (also known as hematuria) or trouble with erections (National Cancer Institute, 2008). Early detection of prostate cancer involves both a Prostate Specific Antigen (PSA) test and Digital Rectal Exam (DRE) and can be further

confirmed by biopsy. If a high risk of prostate cancer is determined, localized treatment such as surgery usually combined with androgen ablation may be utilized while monitoring tumor growth, PSA levels and determining possible metastasis. In cases where tumor growth is not completely removed or a relapse occurs, the cancer becomes more aggressive, leading to androgen-independent or hormone refractory prostate cancer. Advanced cancers may be treated with radiation, chemotherapy or a combination of treatments however, there are many side effects to these options (American Cancer Society, 2008).

Dietary nutrients and nutraceuticals have been targeted in the treatment, prevention or regression of prostate cancer. Food components such as curcumin, resveratrol, indole-3-carbinol, ellagic acid, genistein, capsaicin, ursolic acid, various flavonoids and carotenoids have been targeted as potential dietary agents for the prevention of cancer and other diseases (Aggarwal and Shishodia, 2006). Several studies have shown lutein possesses promising anti-carcinogenic properties and has been shown to have disease preventing properties in various eye, skin and heart diseases (Ribaya-Mercado and Blumberg, 2004; Calvo, 2005; Gunasekera et al, 2007; Mares-Pearlman et al, 2002). Lutein is a yellow, lipophilic oxycarotenoid that is not synthesized by the human body but can be consumed mainly from dark green leafy vegetables, fruits such as kiwi and oranges and from egg yolks (Alves-Rodrigues and Shao, 2003; Calvo, 2005). Lutein is composed of 40 carbon atoms, 56 hydrogen atoms and 2 oxygen atoms and has three chiral-carbons that are enantiomeric. Lutein and zeaxanthin are the only two carotenoids present in the macula, retina and lens region of the eye (Yeum et al, 1995), although

lutein can also be found in other tissues. For example, lutein metabolites can be found in the prostate, colon and lungs with the highest concentration in the liver (Khachik et al, 2002). Lutein is primarily consumed through fruits and vegetables with bioavailability being dependant on the form of food consumed. For example, one study demonstrated an increased bioavailability of lutein in juice form compared to raw vegetables (McEligot et al, 1999).

Lutein has been studied extensively for its health benefits and protection against age-related macular degeneration, cataracts, heart disease, for skin health and various cancers including breast, lung, colon and prostate cancers (Ribaya-Mercado and Blumberg, 2004; Alves-Rodrigues and Shao, 2003; Calvo, 2005). *In vitro* studies have suggested the anti-inflammatory properties of lutein as demonstrated by the decrease in iNOS in mouse macrophage cell lines (Rafi and Shafaie, 2007). *In vivo* studies have also shown lutein demonstrating immunosuppressant and anti-inflammatory properties by decreasing ROS generation following UV exposure (Lee et al, 2004). Additionally, epidemiological studies suggest that consumption of fruits and vegetables rich in lutein are inversely proportional to the incidence of prostate cancer (McCann et al, 2005; Zhang et al, 2007). Although numerous studies have focused on lutein and its effects against prostate cancer, no mechanism of action has been determined.

Our objective is to determine the effect of lutein on gene expression of human prostate cancer biomarker genes in androgen independent human prostate cancer PC-3 cell lines using Oligo GEarray® DNA Microarrays. Human Prostate Cancer Biomarker Genes

microarray contains 263 biomarker genes involved in the development and progression of prostate cancer. Microarray results indicate that treatment of PC-3 cells with lutein significantly down-regulated epidermal growth factor receptor (EGFR), insulin-like growth factor 1 receptor (IGF1R), breast cancer gene 1 (BRCA1), kallikrein 14 (KLK14), prostate cancer antigen 3 (PCA3) and cyclin-dependant kinase 5 (CDK5), while up-regulating ras association domain family 1 (RASSF1) and glutathione s-transferase pi 1 (GSTP1) genes. Results were validated by Real-Time PCR which was consistent with results obtained by microarrays. Western blot analysis also demonstrated the down-regulation of IGF1R and EGFR and up-regulation of GSTP1 at the protein level, suggesting that lutein may be beneficial in preventing or delaying the progression or onset of prostate cancer by modulating specific genes expressed in the disease.

Chapter II

Literature Review

II.A. Carotenoids

Carotenoids are a family of phytochemicals that consist of over 600 structural variations (Rao and Rao, 2007; Fraser and Bramley, 2004). In human plasma, the most dominant carotenoids include β-carotene, lycopene, lutein, β-cryptoxanthin and α-carotene (Rock, 1997). The most abundant sources of these carotenoids are found in a variety of fruits and vegetables. For example, β-carotene can be found in high concentrations in cooked carrots, cantaloupe, broccoli and spinach (Rock, 1997) while lycopene is found predominantly in tomatoes and tomato products (Krinsky and Johnson, 2005). Orange juice, tangerines and peaches contain β-cryptoxanthin (Rock, 1997), while lutein can be found in spinach, kale, broccoli, corn, kiwi, egg yolks and green beans (Rock, 1997; Alves-Rodrigues and Shao, 2003).

Carotenoids have been studied extensively and have been known to improve gap junction communication, pro-vitamin A activity, antioxidant function, immune function, xenobiotic and drug metabolism (Rao and Rao, 2007). Several studies have reviewed the potential health benefits of carotenoids in various diseases including cancer, cardiovascular disease, stroke, HIV and eye diseases like age-related macular degeneration and cataracts (Rao and Rao, 2007; Ribaya-Mercado and Blumberg, 2004; Krinsky and Johnson, 2005).

Carotenoids are isoprenoids that are distinguished by long polyene chains and may consist of 3 to 15 conjugated double bonds (Fraser and Bramley, 2004). Xanthophylls, a sub-family of carotenoids, are formed from hydrocarbon carotenes by the introduction of oxygen atoms (Fraser and Bramley, 2004). The oxygen atoms are paired with a hydrogen atom to form hydroxyl groups which are located on the cyclic ring portions of the structure and are responsible for the increased polarity of lutein and zeaxanthin (Mares-Perlman et al, 2002). Another distinguishable feature of lutein and zeaxanthin is their inability to convert to vitamin A (Mares-Pearlman et al, 2002) which can be attributed to the lack of cleavage by 15,15' monooxygenase enzyme (Alves-Rodrigues and Shao, 2003).

II.B. Lutein Structure

Lutein and zeaxanthin are structural isomers and both occur naturally as *all-trans* compounds (Calvo, 2005), with the difference in structure being attributed to the location of one double bond. Due to the presence of hydroxyl groups on the two terminal beta-ionone rings, lutein has the ability to be esterified with long chain fatty acids in plants (Calvo, 2005). The structure of lutein is shown below.

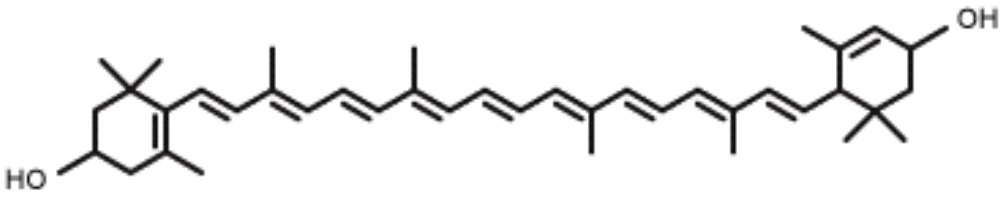


Figure 1. Structure of lutein

II.C. Lutein Content in Food

Lutein is a yellow-orange, lipophilic xanthophyll whose metabolites can be found in various human tissues as well as in human blood and milk (Khachik et al, 2002; Mares-Pearlman, 2002), although lutein is primarily consumed through fruits and vegetables. Some of the most common lutein-rich foods include spinach, broccoli, kale, egg yolk, alfa-alfa, zucchini, corn, peas, brussel sprouts and kiwi (Alves- Rodrigues and Shao, 2003; Krinsky and Johnson, 2005) and the cheapest source of lutein is marigold flowers, however lutein content in these flowers may vary significantly from cultivar, location and season. Lutein attained GRAS status by the FDA in 2003 and is permitted by the European Union as a food coloring agent (European Parliament and Council Directive, 1994). Lutein can also be used as a food additive with an E number of E161b to enhance the color of egg yolk imparting yellow to orange colors in vegetable oils, mayonnaise and dairy products. The lutein contents of various foods are listed below.

Lutein Content in Various Foods


Raw kale: 39.5


Spinach: 11.9


Lettuce: 2.6


Broccoli: 2.4


Summer Squash: 2.1


Corn: 1.8


Peas: 1.4


Okra: 0.4


Egg Yolk: 0.3


Orange: 0.2

*Note: Lutein content in mg/100g wet weight

Figure 2. Lutein content in foods (Krinsky and Johnson, 2005; Alves-Rodrigues and Shao, 2003)

II.D. Bioavailability, Absorption and Metabolism

The bioavailability of carotenoids, including lutein, is influenced by the cellular matrix in which it is integrated, the amount consumed and the method of preparation (Fraser and Bramley, 2004). Cooking in the presence of fats and oils for example, allows increased solubility due to the lipophilic nature of lutein and chopping aids to release lutein from the food matrix, therefore making the method of processing an important factor in determining bioavailability (Alves-Rodrigues and Shao, 2003). Lutein also appears to be less bioavailable in raw or cooked fruits and vegetables as compared to the juice form (McEligot et al, 1999). In one study, researchers found that the bioavailability of lutein from eggs is higher than from spinach, lutein supplements or lutein ester supplements (Chung et al, 2004). The same study suggested that the significantly higher serum lutein response from egg consumption can be attributed to the presence of cholesterol, triglycerides, phospholipids or the fatty acid profile in egg yolk (Chung et al, 2004) which gratifies the lipophilic nature of lutein. Other factors that influence bioavailability include age, amount of alcohol consumed, cigarette smoking, various disease states and overall malnutrition (Alberg, 2002; Albanes et al, 1997; Brady et al, 1996; Alves-Rodrigues and Shao, 2003). Even though there are numerous non-dietary factors influencing bioavailability, various dietary factors including the interaction between other carotenoids or nutrients and lutein need to be considered. There are, however, several inconsistencies among studies and the topic is currently under debate (Alves-Rodrigues and Shao, 2003; Albanes et al, 1997).

Lutein exists in various forms in food and supplements and these forms include lutein esters, pure crystalline lutein and lutein diesters, all of which have varying bioavailability and absorption characteristics. For example, prior to the absorption of lutein diesters, fatty acids must be removed by lipases and esterases (Alves-Rodrigues and Shao, 2003) whereas lutein esters may require de-esterification by intestinal enzymes (Calvo, 2005). Esterification may affect bioavailability although the topic is still under debate (Alves-Rodrigues and Shao, 2003; Bowen et al, 2002).

Following the absorption by mucosal cells in the small intestine, carotenoids are transported through the enterocyte, incorporated into chylomicrons, and then reach the blood to be circulated by HDL and LDL carriers (Alves-Rodrigues and Shao, 2003; Yeum and Russell, 2002). O'Neil and Thurnham (1998) studied the intestinal absorption of carotenoids in human subjects and found that lutein was transported more rapidly from the triacylglycerol-rich lipoprotein (TRL) fraction into other serum lipoproteins as compared to β-carotene and lycopene. For example, the peak concentration of lutein was observed 2 hrs after food consumption while the peak concentrations of β-carotene and lycopene occurred between 4 hrs to 6 hrs. In a study by Park et al (1998), BALB/c mice were utilized to study dietary lutein absorption using lutein esters isolated from marigold flowers. The study found that lutein uptake was rapid in the plasma, liver and spleen of mice where it reached its maximal concentration in the first three days (Park et al, 1998). By day seven, lutein concentrations in plasma had plateaued while accumulation continued in the liver and spleen (Park et al, 1998). The concentration of lutein metabolites in the human body is shown below.

Average Concentration of Various Carotenoids in the Human Body

Dietary carotenoids and their metabolites	Average concentration (ng/g) of carotenoids and their metabolites in human tissues[a] and skin[b]						
	Liver (n = 3)	Lung (n = 3)	Breast (n = 3)	Cervix (n = 3)	Prostate (n = 5)	Colon (n = 3)	Skin (n = 3)
Dietary carotenoids							
α-carotene	67	47	128	23.6	50	128	8
β-carotene + Z-isomers	470	226	356	125.3	163	256	26
γ-carotene	–[c]	–[c]	–[c]	–[c]	48	–[c]	20
Lycopene	352	300	234	95.0	374	534	69
ζ-carotene	150	25	734	57.2	187	134	13
Phytofluene	261	195	416	106.3	201	116	15
Phytoene	168	1275	69	–[c]	45	70	65
α-cryptoxanthin	127	31	23	4.0	32	21	–[c]
β-cryptoxanthin	363	121	37	24.3	146	35	–[c]
Lutein + Z-isomers	1701	212	90	23.8	128	452	26
Zeaxanthin + Z-isomers	591	90	14	–[c]	35	32	6

Table 1. Concentration of lutein in human tissue and skin with highest concentration in liver followed by colon, lungs and prostate (Khachik et.al, 2002).

II.E. Recommended Intake and Toxicity

The average daily intake of lutein in the United States is approximately 2 mg according to the USDA (USDA Agricultural Research Service, 2004), although no recommended intake has been established. Human intervention studies have supplemented patients with varying concentrations of lutein over any given number of days and thus far, no adverse effects have been reported (Alves-Rodrigues and Shao, 2003). For example, in a study by Berendschot et al (2000), male patients with a mean age of 40.6 yrs were supplemented with 10 mg of lutein for 12 weeks and found that the concentration of lutein in the plasma was correlated with a higher macular pigment density. In another study, Landrum et al (1997), supplemented subjects with 30 mg of lutein for 140 days and observed similar results regarding macular pigment density. No adverse effects have been reported with high doses of lutein for extended periods of time although one reversible side effect that has been documented is the incidence of carotenodermia which is a hyperpigmentation of

the skin due to prolonged consumption of carotenoids (Alves-Rodrigues and Shao, 2003; Sies and Stahl, 2004).

II.F. Health Benefits of Lutein

The health benefits of lutein have been studied in protecting against diseases such as cataracts, age-related macular degeneration, cancers, heart disease and stroke (Ribaya-Mercado and Blumberg, 2004; Alves-Rodrigues and Shao, 2003) although lutein has gained most of its popularity due to its protective role in eye health. Two ocular tissues that play a major role in vision are the macula and the lens and coincidentally of all the carotenoids present in human serum, lutein and zeaxanthin are the only ones present in these tissues (Yeum et al, 1995). Krinsky and Johnson (2005) proposed a biological mechanism for the protective role of lutein which included its function as an antioxidant and its ability to filter out harmful blue light while another study focused on the free radical scavenging properties of lutein (Beatty et al, 2000). This was further demonstrated by the decreased lipid peroxidation in the presence of lutein even though the macula has a high concentration of polyunsaturated fatty acids that are susceptible to oxidative damage (Beatty et al, 2000; Alves-Rodrigues and Shao, 2003). Researchers have even discovered and identified Xanthophyll Binding Protein (XBP) in the human eye which is a protein that is highly specific to lutein (Alves-Rodrigues and Shao, 2003).

II.F.1. Lutein and Eye Health

Age-related macular degeneration (AMD) is a condition in which there is a loss of vision or blindness in the central portion of the eye caused by retinal damage which usually

affects people aged 65 and older (Alves-Rodrigues and Shao, 2003). Factors that contribute to the onset of AMD include oxidative stress, age, sunlight exposure, smoking and malnutrition (Beatty et al, 2000; Alves-Rodrigues and Shao, 2003). Investigators have implicated lutein as having a protective role against AMD with this notion being supported by many human studies including case-control studies and analysis of tissue and plasma lutein concentrations following supplementation (Ribaya-Mercado and Blumberg, 2004; Alves-Rodrigues and Shao, 2003; Berendschot et al, 2000; Johnson et al, 2000).

II.F.2. Lutein and Heart Disease

There has been growing evidence suggesting that lutein may play a role in the prevention of coronary heart disease. For example, in The Los Angeles Atherosclerosis Study by Dwyer et al (2001), 480 middle-aged men and women were administered with lutein over a period of 18 months and tested for the progression of intima-media thickness (IMT). Measurements of lutein concentrations in plasma ranged from 20 nmol/l - 930 nmol/l, with results showing a lack of progression of IMT in the highest quintile as compared to the lowest quintile. Subjects with at least 100 nmol/l of plasma lutein also demonstrated the inhibition of monocyte migration (Dwyer et al, 2001). These findings were further tested in apoE-null mice by supplementation of lutein and resulted in reduced aortic lesion size as compared to the control group (Dwyer et al, 2001). Another study demonstrated that lutein-treated endothelial cells significantly decreased the expression of adhesion molecules such as VCAM-1, E-selectin and ICAM-1, indicating a reduction

in inflammatory stimuli that are known to contribute to cardiovascular disease (Martin et al, 2000).

II.F.3. Lutein and Skin Health

There is evidence demonstrating the role of lutein in maintaining skin health by reducing the incidence of UV damage (Alves-Rodrigues and Shao, 2003) as well as its role in reducing erythema caused by UV damage when coupled with lycopene and β-carotene supplementation (Sies and Stahl, 2004). *In vivo* studies demonstrated that lutein supplementation leads to decreased UV-induced inflammation and reduces cellular damage by decreasing ROS formation in mice (Lee et al, 2004). Another study found that the combined oral and topical administration of lutein leads to decreased skin lipid peroxidation, improved skin elasticity and increased skin hydration in clinical trials (Palombo et al, 2007).

II.F.4. Lutein and Cancer

Lutein has been identified as one of the antimutagenic pigments present in extracts of Porphyra tenera which is an edible seaweed known for its suppressive activity in mutagenesis and possibly carcinogenesis (Okai et al, 1996). Lutein has also demonstrated chemopreventative activity in animal models for colon (Kim et al, 1998) and breast cancers (Park et al, 1998). Specifically, Kim et al (1998) demonstrated the inhibition of abberant crypt foci in mice colons following treatment with lutein while Park et al (1998) observed an inhibition of mammary tumor growth, lower tumor weight and longer tumor latency period in BALB/c mice following 0.002% lutein treatment. Park et al (1998) also

suggested the role of lutein to include the prevention of tumor initiation. Human studies have demonstrated that diets rich in fruits and vegetables, especially lutein, may reduce the risk for laryngeal cancer (Bidoli et al, 2003), lung cancer but by only 10-19% (Michaud et al, 2000), breast cancer in pre-menopausal women (Hulten et al, 2001) and prostate cancer (McCann et al, 2005). This is possibly due to the role of lutein as an antioxidant, a chemopreventative agent or as a regulator of apoptosis, angiogenesis and gap junctional intercellular communication (Ribaya-Mercado and Blumberg, 2004).

II.G. Cancer in the United States

Cancer is the second leading cause of death (22.8% of all deaths) following heart disease (26.6%) (American Cancer Society, 2008) and accounts for 1 death of every 4 deaths in the United States. According to the American Cancer Society, in 2008 lung cancer will account for approximately 31% of cancer related deaths in males, followed by prostate cancer (10%), colon and rectum (8%) and pancreatic cancer (6%) while the leading sites of cancer death in women are lung (26%), breast (15%), colon and rectum (9%) and pancreas/ovaries (6%). Approximately 295,000 males and 272,000 females are expected to be victims of cancer related deaths in the US this year with new cases of cancer expecting to reach 1,437,180 this year alone (American Cancer Society, 2008).

Cancer is a disease that is characterized by uncontrolled cell growth and can be a result of external factors including tobacco use, chemicals, radiation, and diet and internal factors including hormones, immune conditions, age, race and genetic factors (American Cancer Society, 2008). In fact, about 5% of cancers are due to genetic factors including somatic

mutations and inherited genes (American Cancer Society, 2008). In 2008 The American Cancer Society also estimates that out of the expected 565,650 cancer deaths, about one-third of the cases will be related to nutritional status, activity level and/or excess weight while tobacco use will be responsible for about 170,000 of those cancer deaths.

II.H. Prostate Cancer

The prostate is an accessory gland in the male reproductive system and is responsible for aid in sperm function (Clinton and Giovannucci, 1998). It is located in front of the rectum, below the urinary bladder and wraps around the urethra (American Cancer Society, 2008; Clinton and Giovannucci, 1998). The prostate begins developing during fetal development due to the secretion of testosterone and continues to grow through adulthood with a rapid growth during puberty (Clinton and Giovannucci, 1998). In some adult males, a portion of the prostate continues to grow with age, eventually causing BPH (Benign Prostatic Hyperplasia) which should not be confused with prostate cancer (American Cancer Society, 2008; Clinton and Giovannucci, 1998). Symptoms associated with prostate cancer that may parallel some of the symptoms of BPH include an inability to urinate, weak urine flow, painful urination, presence of blood in urine (also known as hematuria) or trouble with erections (National Cancer Institute, 2008; American Cancer Society, 2008). Some of these side effects are partly due to the close proximity of the prostate to the bladder and urethra, which becomes constricted once prostatic cells grow and multiply locally.

There are currently no definite causes of prostate cancer however many risk factors have been identified. Age, for example, is the strongest risk factor with 2 out of 3 prostate cancer incidences found in men over the age of 65 (American Cancer Society, 2008). Prostate cancer is also more prevalent in African American men than Caucasian or Hispanic men with even fewer cases in Asian American men (Clinton and Giovannucci 1998; American Cancer Society, 2008). Aside from age and race, family history has also been associated with an increased risk of prostate cancer especially if a close family member was affected (Clinton and Giovannuci, 1998; American Cancer Society, 2008). Studies also show that diet is an important risk factor with an increased risk in men who consume less fruits and leafy green vegetables (Cohen et al, 2000).

II.I. Prostate Cancer Screening and Stage Classification

The presence of a tumor often leads to classification of prostate cancer into four different stages for the purpose of determining the best treatment options and overall likelihood of survival (American Cancer Society, 2008). Stage I represents the initial stage in which less than 5% of prostate tissue is cancerous while Stage II is defined by the increased number of localized cancerous cells detectable by Digital Rectal Exam (DRE) and/or increased Prostate Specific Antigen (PSA) levels (American Cancer Society, 2008). A PSA level of 4 ng/ml or below is considered normal (Garnick and Fair, 1998) but if PSA levels are in between 4 ng/ml and 10 ng/ml, there is approximately a 25% chance of having prostate cancer and chances almost double with a PSA level greater than 10 ng/ml (American Cancer Society, 2008). Since some cancer patients can have normal PSA levels, it is recommended that PSA and DRE tests be performed in conjunction and

confirmed by a biopsy (American Cancer Society, 2008). If the biopsy is positive, a Gleason score is assigned to grade the extent of prostate cancer with a Grade 1 Gleason score being used to describe normal prostate tissue and a score of 5 assigned to cancerous cells that are scattered throughout the prostate (American Cancer Society, 2008). A maximum Gleason score of 10 is assigned to cancers that are considered high-grade or for those tumors that are likely to metastasize more rapidly (American Cancer Society, 2008).

Stage III prostate cancer is determined by the presence of cancerous cells in organs and tissues of close proximity (American Cancer Society, 2008) and is considered to be the beginning of an advanced and more aggressive form of prostate cancer. Metastasis, or Stage IV, is defined by the spread of cancerous cells into organs and tissues beyond the prostate region leading to an approximate cancer survival rate of 37% (American Cancer Society, 2008).

II.J. Treatment of Prostate Cancer

Treatment of prostate cancer includes surgery, radiation therapy, cryosurgery, hormone therapy, chemotherapy and alternative/complementary medicine but these treatment options can be influenced by a number of factors including age, health conditions, stage of cancer, side effects of treatment options and personal choice (American Cancer Society, 2008). Surgery to remove the tumor is often used in cancers that remain within the prostate although there are many side effects that would need to be considered by the patient including possible urinary incontinence or impotence (American Cancer Society,

2008). Radiation therapy, on the other hand, is used for patients whose cancer is found locally or has spread minimally to surrounding tissue although side effects can include bowel and bladder problems, difficulty urinating, impotence, exhaustion and lymphedema (American Cancer Society, 2008). Radiation therapy is also used for patients with recurring cancer and can be used to decrease tumor size for temporary relief (American Cancer Society, 2008). Hormonal therapy is another option that is generally used to shrink, destroy or impede the growth of cancer cells that other treatments may have missed (American Cancer Society, 2008; Garnick and Fair, 1998). This form of therapy involves reducing testosterone and dihydrotestosterone with consequences including fatigue, loss of bone density, impotence, hot flashes and loss of sexual drive (Garnick and Fair, 1998; American Cancer Society, 2008). Another form of treatment known as chemotherapy involves the use of anti-cancer drugs which are either injected or taken orally and are then able to circulate within the body (American Cancer Society, 2008). This treatment can be used for metastasized cancer however the major disadvantages can include loss of hair, loss of appetite, weight loss, fatigue, nausea, vomiting and a weakened immune system (American Cancer Society, 2008).

II.K. Prostate Cancer Biomarker Genes

Proto-oncogenes and tumor suppressor genes play a major role in homeostasis by regulating cell proliferation and apoptosis (Goldsby et al, 2003). Proto-oncogenes encourage cell proliferation by encoding proteins that can function as growth factors or receptors as part of a highly regulated system (Goldsby et al, 2003). However, a mutation or inappropriate expression of these growth factors can de-regulate the cell proliferation

process ultimately causing uncontrolled cell growth (Goldsby et al, 2003). Tumor suppressor genes, on the other hand, are responsible for inhibiting excessive cell growth so when these genes are inactivated or mutated they can cause uncontrolled cell proliferation (Goldsby et al, 2003).

Researchers are studying gene expression in prostate cancer to better evaluate aggressive tumors and biomarker genes that are involved in proliferation, angiogenesis, apoptosis and metastasis (Bull et al, 2001). Gene-based research focuses on the analysis of hundreds of genes which allows for a more accurate assessment of prostate cancer (Bull et al, 2001). The analysis of these biomarker genes coupled with research from validation studies, epidemiological data and studies of protein expression can offer a deeper look into factors affecting prostate cancer progression.

II.K.1. Epidermal Growth Factor Receptor (EGFR)

Epidermal growth factor receptor (EGFR) is a 170 kDa membrane spanning protein, whose overexpression has been associated with aggressive tumors thereby making it a common target for anti-cancer therapy (Zandi et al, 2007). For instance, one review demonstrated a correlation between increased EGFR expression and the reduction of overall cancer survival rate (Nicholson et al, 2001) while another study implicated EGFR and its role in cell motility, invasion, inhibition of apoptosis, angiogenesis and metastasis (Herbst and Shin, 2001). Elevated EGFR expression has also been identified in the promotion and aggressiveness of tumors in multiple cancer types including prostate,

cervical, breast, pancreatic, head and neck, bladder, colon, ovarian and oesophageal cancers (DiLorenzo et al, 2002; Nicholson et al, 2001; Herbst and Shin, 2001).

II.K.2. Insulin Like Growth Factor 1 Receptor (IGF1R)

IGF1R is a transmembrane heterodimer that is a part of the insulin receptor family and has tyrosine-kinase receptor activity (Larsson et al, 2005; Happerfield et al, 1997). IGF1R promotes tumor survival and tumorigenesis (Cohen et al, 2004) and plays a role in malignant transformation (Miller and Yee, 2005), cell motility, proliferation and cancer cell survival (Sachdev and Yee, 2006).

II.K.3. Breast Cancer Gene 1 (BRCA1)

BRCA1 is a tumor suppressor gene located on chromosome 17q21 but upon mutation this gene has been linked to hereditary breast cancer (Dong, 2006). Other studies have also associated the overexpression of mutated BRCA1 with an increased risk for ovarian, colon, pancreatic, cervical and prostate cancers (Ford and Easton, 1994; Thompson and Easton, 2002). Specifically, Mitra et al (2008) suggested that BRCA1 and BRCA2 mutations are markers for a more aggressive prostate cancer. The same study also mentioned that BRCA1 and BRCA2 mutation carriers under the age of 65 tend to have a higher incidence of prostate cancer and a higher Gleason score than non-carriers (Mitra et al, 2008).

II.K.4. Cyclin Dependant Kinase 5 (CDK5)

CDK5 is known for its role in neuronal degeneration, neuronal apoptosis (Lin et al, 2004) and as a migration regulator in neuronal development but more recently, it has been shown to possess an important role in prostate cancer cell motility and spontaneous metastasis (Strock et al, 2006). In fact, studies demonstrated that CDK5 is active in prostate cancer cell lines LnCaP, DU145 and PC3 along with its activator, p53 (Lin et al, 2004; Strock et al, 2006). Strock et al (2006) also found that p53 was correlated with 87.5% of metastasized prostate cancer cases after evaluating microarrays representing tissue samples of 32 patients.

II.K.5. Kallikrein 14 (KLK14)

KLK14 is located on chromosome 19q13.4 and is an extracellular serine protease belonging to the human tissue kallikrein family (Borgono et al, 2006). KLK14 is believed to be differentially expressed in prostate cancer tissues and was even found to be up-regulated by androgens in cancer cells (Yousef et al, 2003). The up-regulation of KLK14 has been associated with advanced and aggressive tumors and its protein, hK14, has the potential to be used as an indicator of prostate cancer in the future (Yousef et al, 2003).

II.K.6. Prostate Cancer Antigen 3 (PCA3)

PCA3, a non-coding RNA, is over expressed in prostate cancer and is highly specific to tumor cells (van Gils et al, 2007). One study found that the higher the expression of PCA3 in the urine, the greater the likelihood of a positive biopsy (Haese et al, 2008)

while another study correlated increased tumor volume with an increased PCA3 score (Whitman et al, 2008).

II.K.7. Ras Association Domain Family 1 (RASSF1)

Tumor suppressor gene RASSF1, located on chromosome 3p21.3, has been commonly implicated as a regulator of cell cycle progression for the transit during G1/S phase (Whitehurst et al, 2008). RASSF1A, a major splice form of RASSF1, has also been shown to induce cell cycle arrest and inhibit cell proliferation, ultimately inhibiting tumorigenesis (Shivakumar et al, 2002). However, in prostate cancer, RASSF1 can experience epigenetic inactivation or silencing by undergoing promoter CpG island methylation and consequently lose its tumor suppressor function (Li et al, 2005; Agathanggelou et al, 2005)

II.K.8. Glutathione S-Transferase Pi 1 (GSTP1)

GSTP1 is a phase II detoxifying agent that inactivates carcinogenic compounds (Obligacion et al, 2006; Li et al, 2005; Cohen et al, 2000) and is considered to be the most abundant GST in the human prostate (Cohen et al, 2000). However, GSTP1 is often inactivated or silenced due to the hypermethylation of the CpG islands allowing for the progression and development of prostate cancer (Obligacion et al, 2006; Li et al, 2005).

II.L. Prostate Cancer and Lutein

There are many genes that are involved in cancer including those that have the ability to influence the regulation of hormonal or cell cycle pathways, DNA repair, immune or

neurotransmitter function, nutrient metabolism and the regulation of oncogenes and tumor suppressor genes (Sinha et al, 1999; Greenwald, 2001). To develop effective approaches against cancer, it is essential to understand gene-nutrient interactions and its involvement in cancer progression or prevention (Greenwald, 2001). Lutein is one of the nutraceuticals that has been reviewed extensively in epidemiological studies and has been the focus of many *in vitro* and *in vivo* studies for its effect against a range of cancers, including prostate cancer.

In vitro studies have suggested that dietary lutein may act as an anti-inflammatory agent. For example, in a study by Rafi and Shafaie (2007), the anti-inflammatory effect of lutein using LPS-stimulated mouse macrophage cell lines were explored to obtain a better understanding of the molecular mechanism of action. Results demonstrated a 50% decrease in the production of LPS stimulated nitric oxide as compared to the control as well as a reduction in iNOS expression at the mRNA and protein levels. Findings suggested the anti-inflammatory properties of lutein as demonstrated by the decrease in iNOS in mouse macrophage cells (Rafi and Shafaie, 2007). Another study suggested carotenoids including lutein, inhibit cell proliferation and induce cell cycle arrest in PC-3 and LNCaP cell lines (Lu et al, 2005). In a study by Gunasekera et al (2007), lutein was shown to inhibit malignant AT3 rat prostate carcinoma cells in a concentration and time-dependant manner with a 42% inhibition of the lutein treated cells on the fourth day. In addition, *in vivo* studies have also demonstrated anti-inflammatory and immunosuppressant properties in mice, showing a reduction in reactive oxygen species (ROS) following lutein consumption (Lee et al, 2004).

Epidemiological studies often demonstrate a reduced risk of prostate cancer associated with high vegetable consumption. For example, a population-based case controlled study in Seattle, WA recorded and tracked the diets of 628 men under the age of 65 who were newly diagnosed with prostate cancer as well as the diets of 602 men as a control. Diets were assessed over 3-5 years and results showed that a high consumption of cruciferous vegetables was inversely proportional to the risk of prostate cancer (Cohen et al, 2000). Diets that included at least 2000 μg of lutein consumption also showed a 32% reduced risk of prostate cancer (Cohen et al, 2000). Another population-based case-controlled study in western New York showed similar results by demonstrating a reduced risk of prostate cancer for men in the highest quintile of lutein intake (McCann et al, 2005).

Chapter III

Hypothesis and Objectives

III.A. Hypothesis

If lutein modulates the expression of human prostate cancer biomarker genes involved in cell proliferation, differentiation, angiogenesis or apoptosis, then lutein may delay or prevent progression of prostate cancer.

III.B. Objectives

III.B.1. To determine the effect of lutein on cell viability in hormone refractory PC-3 line using MTT cell viability assay

III.B.2. To investigate the effect of lutein on 263 biomarker genes involved in prostate cancer using Oligo GEArray® DNA Microarray technology

III.B.3. To validate the expression of modulated genes at the transcription level using Real-Time PCR

III.B.4. To investigate protein expression of Insulin-like Growth Factor 1 Receptor (IGF1R), Epidermal Growth Factor Receptor (EGFR) and Glutathione S-Transferase Pi 1 (GSTP1) using Western Blot

Chapter IV

Materials and Methods

IV.A. Cell Culture

Hormone-refractory human prostate cancer cell line (PC-3) was obtained from American Type Culture Collection (ATCC- Manassas, Virginia), cultured on 100 mm tissue culture plates and held in an incubator at 37°C and 5% CO_2. PC-3 cells were supplemented with 500 mL RPMI-1640 Medium (Gibco, Carlsbad, CA), 10% Fetal Bovine Serum (Gemini Bio-products, West Sacramento, CA) and 1% Penicillin/Streptomycin Antibiotics (Gibco, Carlsbad, CA). Lutein was purchased from Sigma-Aldrich (St. Louis, MO).

IV.B. MTT Cell Viability Assay

MTT (3-[4, 5 dimethyl-thiazol-2-yl]-2, 5-diphenyltetrazolium bromide), a pale yellow tetrazolium salt, was used to measure cell viability. In living and metabolically active cells, this yellow substrate reduces to form a dark blue/purple formazan product with the number of living cells being directly proportional to the intensity of formazan product created. PC-3 cells were cultured in a sterile 96-well plate (200 µL per well) and incubated at 37ºC. After 48 hrs of incubation, cells were treated with lutein in concentrations ranging from 2.5 µM to 50 µM for 22 hrs. Media was aspirated and 100 µL MTT dye (1 mL MTT dye in 9 mL media) was added. Following an incubation of 2.5 hrs, 200 µL of culture medium was aspirated from each well and a mixture of 14.85 mL of isopropanol with 150 µL HCl was added to dissolve the insoluble purple formazan product. The acidified cell culture was incubated for 30 min at 37ºC. Cells were then

resuspended in solution and optical density was measured at 570 nm, using ELISA micro plate reader (BioRad Microplate Reader 680, BioRad Laboratories, California).

IV.C. RNA Isolation

Cells were treated with 10 μM of lutein, a concentration established by MTT cell viability assay, for 22 hrs at 37ºC. RNA was isolated from untreated (control) and 10 μM lutein treated PC-3 cells as per established lab protocol. Treated and untreated cells were washed with Phosphate Buffered Saline (PBS) and centrifuged for 5 min at 4°C and 5,000 rpm. The supernatant was discarded and the pellet was homogenized with Tri-reagent (Sigma-Aldrich, St. Louis, MO) and separated into aqueous and organic phases by the addition of chloroform (Sigma-Aldrich, St. Louis, MO). RNA was precipitated by addition of isopropanol to the aqueous phase and washed with ethanol before solubilization. Qualitative analysis of RNA was performed using a spectrophotometer and measured at the 260/280 wavelength ratio to obtain an optimal reading between 1.6 and 2.0. Quality was also studied using separation of ethidium bromide stained RNA in agarose gel.

IV.D. Oligo GEArray® DNA Microarray

RNA of treated and untreated cells were extracted, reverse transcribed to cDNA, labeled with biotin and transcribed to cRNA using True-Labeling AMP^{TM} 2.0 kit (Superarray Bioscience Corporation, Frederick, MD). Biotin-labeled cRNA was further hybridized with Human Prostate Cancer Oligo GEArray® DNA Microarrays for 24 hrs followed by gene detection on x-ray film using a Chemiluminescent Detection kit (Superarray

Bioscience Corporation, Frederick, MD). Arrays were then analyzed with the use of GEArray® Expression Analysis Suite 2.0 software and genes were normalized to GAPDH, a housekeeping gene. Results were calculated and percent changes were obtained between treated and untreated samples.

IV.E. Real-Time Polymerase Chain Reactions (Real-Time PCR)

Quantitative analyses of consistently modulated genes were analyzed using iCycler MYIQ Real time PCR detection system (Bio-Rad, Hercules, CA). Primers and probes for EGFR, IGF1R, BRCA1, KLK14, PCA3, CDK5, RASSF1 and GSTP1 were obtained from Applied Biosystems (Foster City, CA). To detect gene expression, amplification of the gene transcript was needed, which first required the isolated RNA samples to be reverse transcribed to cDNA via the enzyme, reverse transcriptase. Mastermix was prepared by using 200 ng RNA, 1.25 µl TaqMan® reverse-transcriptase enzyme, 25 µl TaqMan® One-step RT-PCR Master Mix (Applied Biosystems, Foster City, CA), 2.5 µl TaqMan® primers and probes, and 19.25 µl RNase free water to obtain a total volume of 50 µl. Parameters for amplification included one cycle for 15 min at 55°C, another cycle at 95°C for 3 min, and forty cycles at 60°C for 30 sec each. Genes EGFR, IGF1R, BRCA1, CDK5, PCA3, KLK14, RASSF1 and GSTP1 were analyzed for both lutein treated and untreated cells and fold differences were calculated using the comparative C_T method ($\Delta\Delta C_T$ method) as per manufacturer's instructions (Applied Biosystems, Foster City, CA). The average C_T value of each sample was calculated and standard deviations were obtained for each average C_T value. ΔC_T values were calculated by subtracting the mean C_T value of GAPDH from the mean C_T values of EGFR, IGF1R, BRCA1, CDK5,

PCA3, KLK14, RASSF1 and GSTP1 for each sample. The $\Delta\Delta C_T$ value was calculated by subtracting the ΔC_T value of 10 μM lutein treated samples minus the ΔC_T value of untreated (control) samples. Fold differences using the $\Delta\Delta C_T$ method are usually expressed as a range due to the incorporation of the standard deviation into the fold difference calculation. The range for target *n*-fold difference is calculated by $2^{-\Delta\Delta CT}$ with $\Delta\Delta C_T + s$ and $\Delta\Delta C_T - s$, where *s* is the standard deviation of the $\Delta\Delta C_T$ value (Applied Biosystems). The values of treated samples were expressed as *n*-fold difference relative to the expression of control samples. Real-Time PCR experiments were performed in triplicates and an average fold difference in gene expression between lutein treated and untreated samples was calculated. GAPDH, a housekeeping gene, was used as a calibrator to determine accurate gene modulations between samples.

IV.F. Protein Isolation

PC-3 cells treated with 10 μM lutein or supplemented solely with RPMI media, were washed twice with Phosphate Saline Buffer (PBS) after a 22 hr incubation period. Cold lysis buffer was added for solubilization and lysates were centrifuged at 12,500 rpm for 30 min at 4°C. Supernatant was collected and protein was estimated using the Bradford method.

IV.G. Western Blot

PC-3 cells were treated with 10 μM lutein and incubated for 22 hrs. Following incubation, samples were washed with cold PBS and dissolved in cold lysis buffer which contained 10 mM Tris-base, 5 mM EDTA, 5 mM phenylmethylsulfonyl fluoride (PMSF),

50 mM NaCl, 1% Triton X-100, 2 mM sodium orthovanadate, 10 µg/ml leupeptin, 25 µg/ml aprotinin, 1 mM sodium pyrophosphate and 20% glycerol and held on ice for 30 min. Lysates were then centrifuged for 15 min at 4°C at 12,500 rpm and supernatant was collected. Protein concentrations were determined using a spectrophotometer and results were compared to standard protein concentrations. Approximately 100 µg were collected from each sample and loaded onto 10% separating SDS-page gel (polyacrylamide electrophoresis gel) and resolved for 5 hrs at 120 v in running buffer containing 95 mM Tris-HCl, 960 mM glycine, and 0.5% SDS. The proteins were then transferred onto Hybond chemiluminescence nitrocellulose membrane (Amersham Biotech, NJ, USA) over a period of 3 hrs at 200 mA in transfer buffer containing 25 mM Tris-base, 192 mM glycine, and 20% methanol. The nitrocellulose membrane was blocked with 5% nonfat dry milk in PBST comprised of 20 mM Sodium Phosphate buffer, pH 7.6, 150 mM NaCl, 0.1% Tween 20, at room temperature for 1 hr. Primary antibody (Santa Cruz Biotechnologies, Santa Cruz, CA) was added onto the membrane with blocking solution and incubated at 4°C overnight. The membrane was washed 4 times for 10 min each with PBST, followed by the addition of secondary antibody (Santa Cruz Biotechnologies, Santa Cruz, CA) into the blocking solution for 1 hr at room temperature and then washed with PBST 4 times for 5 min each. Membranes were exposed to X-ray film and band density was measured using Bio-rad Quantity One 1D Analysis Software (Bio-rad, Hercules, CA).

Chapter V

Results

V.A. MTT Assay for Cell Viability

To determine the highest non-toxic dose of lutein, PC-3 cells were treated with various concentrations of lutein ranging from 2.5 µM- 50 µM. After 22 hrs of incubation, a cell viability assay was performed using MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide) dye. Optical density was measured at 570 nm to calculate an index of cell viability. Figure 3 below demonstrates the non-toxic effect of lutein at a concentration of 10 µM. PC-3 cells were non-viable at lutein concentrations of 50 µM, 40 µM, 30 µM and 20 µM, but retained their viability at 10 µM, 5 µM and 2.5 µM.

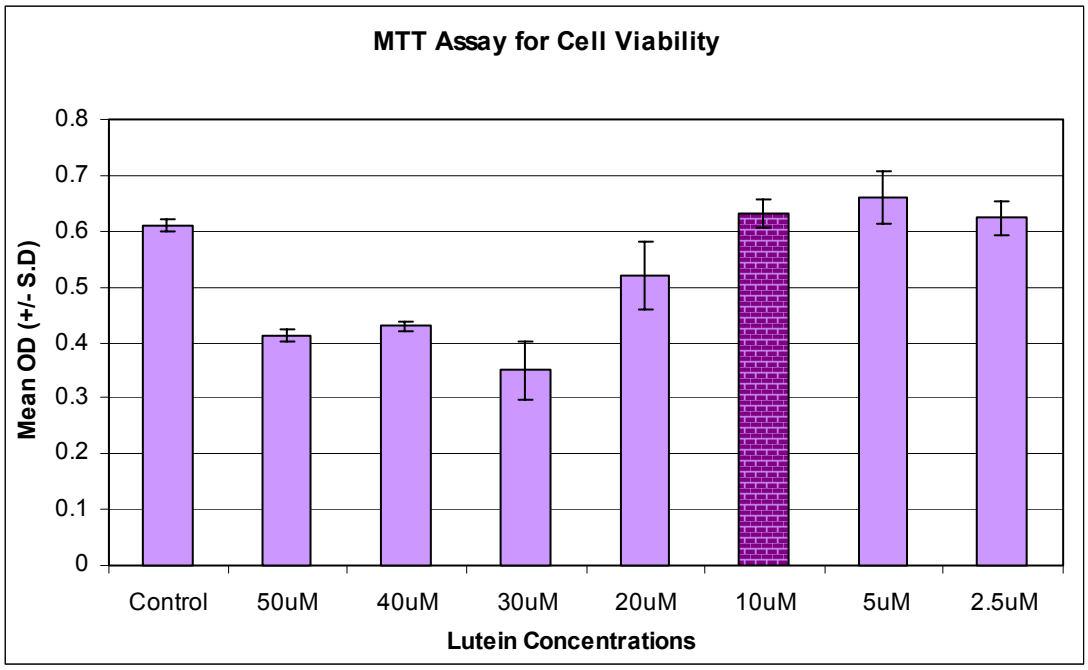


Figure 3. MTT assay for cell viability indicates lutein treatments of 50 µM, 40 µM, 30 µM and 20 µM result in a decrease in cell viability. The highest non-toxic dose was determined to be 10 µM and therefore was used for further experiments.

V.B. RNA Isolation

RNA was isolated from untreated and 10 μM lutein-treated PC-3 cells based on protocol established in our laboratory. RNA quality was determined by 1% agarose gel electrophoresis and RNA quantity was obtained by measuring RNA absorption at 260 nm. Good quality RNA is indicated by an RNA/protein ratio between 1.6 and 2.0 measured by spectrophotometer at a wavelength of 260 nm. RNA quality was indicated by the visible separation of two distinct bands of RNA, 28S RNA and 18S RNA, respectively.

RNA Sample ID	WL260.0	WL280.0	Ratio (260/280)	RNA Concentration in Sample
Control	0.111	0.067	**1.646**	**1.11μg/μL**
10μM lutein	0.150	0.093	**1.623**	**1.50μg/μL**

Table 2.Determination of RNA quantity by spectrophotometer

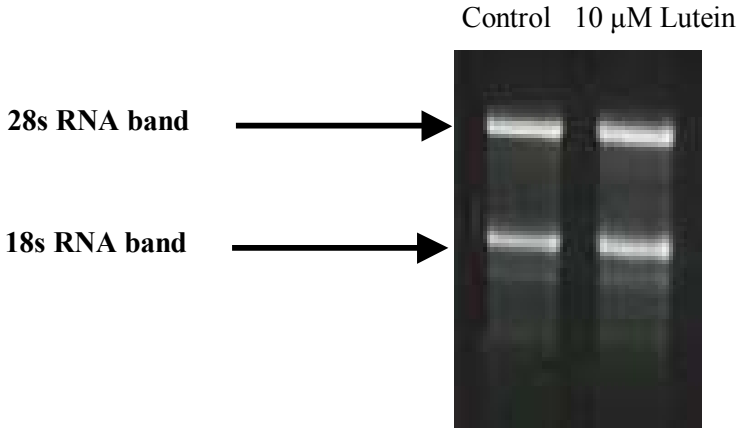


Figure 4. RNA isolated from control (untreated) and 10 μM lutein-treated PC-3 cells. Gel electrophoresis indicates the ideal separation of 28S RNA and 18S RNA bands.

V.C. Oligo GEArray® DNA Microarray

RNA of treated and untreated cells was reverse transcribed to cDNA, labeled with biotin and transcribed to cRNA. Arrays were hybridized with cRNA overnight and gene

expression was detected using chemiluminescence detection on x-ray film. Microarray experiments were performed in triplicates and an average percent modulation was calculated.

Modulation of Prostate Cancer Biomarker Genes

Gene	Average Percent Down-regulation
KLK14	84%
BRCA1	69%
IGF1R	51%
PCA3	50%
CDK5	48%
EGFR	47%

Table 3. Average percent down-regulation of prostate cancer biomarker genes after 10 μM lutein treatment.

Gene	Average Percent Up-regulation
GSTP1	59%
RASSF1	35%

Table 4. Average percent up-regulation of prostate cancer biomarker genes after 10 μM lutein treatment.

Microarrays were performed in triplicates and the percent differences were calculated. The average value of each gene was determined and the above genes represent the most consistently modulated genes among triplicate experiments. The average percent modulation in gene expression between untreated and lutein treated PC-3 cells were calculated as follows:

Average percent modulation =

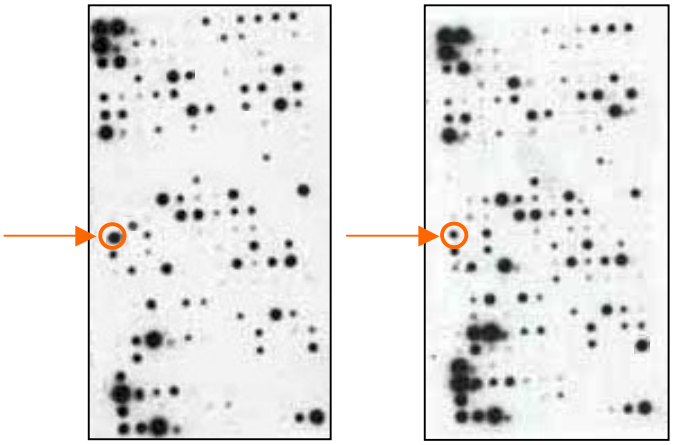
$$\frac{\text{average gene expression (untreated) - average gene expression (10 μM lutein treated)}}{\text{average gene expression (untreated)}} \times 100$$

RPS27A 1	RPS27A 2	AGR2 3	AGTR2 4	AIG1 5	AKAP1 6	AKT1 7	APC 8	APOC1 9	GAPDH 10	GAPDH 11	GAPDH 12
RPS27A 13	AR 14	BAK1 15	BAX 16	BCL2 17	BCL2L1 18	BMP6 19	BRCA1 20	CANT1 21	CASP1 22	CASP3 23	CASP7 24
CAV1 25	CCND1 26	CD164 27	CD44 28	CDH1 29	CDH10 30	CDH12 31	CDH13 32	CDH18 33	CDH19 34	CDH20 35	CDH7 36
CDH8 37	CDH9 38	CDK2 39	CDK3 40	CDK4 41	CDK5 42	CDK6 43	CDK7 44	CDK8 45	CDK9 46	CDKN1A 47	CDKN1B 48
CDKN1C 49	CDKN2A 50	CDKN2B 51	CDKN2C 52	CDKN3 53	CHGA 54	CHGB 55	CLDN3 56	CLN3 57	CLU 58	COL1A1 59	COL6A1 60
CYB5 61	CYC1 62	DAB2IP 63	DAPK1 64	DES 65	DNCL1 66	E2F1 67	EGF 68	EGFR 69	EGR3 70	ELAC2 71	ELL 72
ENO1 73	ENO2 74	ENO3 75	ERBB2 76	ERK8 77	ESR1 78	ESR2 79	EZH1 80	EZH2 81	FASN 82	FGF1 83	FGF10 84
FGF11 85	FGF12 86	FGF13 87	FGF14 88	FGF16 89	FGF17 90	FGF18 91	FGF19 92	FGF2 93	FGF20 94	FGF21 95	FGF22 96
FGF23 97	FGF3 98	FGF4 99	FGF5 100	FGF6 101	FGF7 102	FGF8 103	FGF9 104	FHIT 105	FLJ12584 106	FLJ25530 107	FOLH1 108
GAGEB1 109	GAGEC1 110	GGT1 111	GNRH1 112	GRP 113	GSTP1 114	HIF1A 115	HIP1 116	HK2 117	HK3 118	HRAS 119	HUMCYT2A 120
IGF1 121	IGF1R 122	IGF2 123	IGFBP3 124	IGFBP6 125	IL12A 126	IL1A 127	IL1B 128	IL2 129	IL24 130	IL29 131	ILK 132
INHA 133	INSL3 134	INSL4 135	ITGA1 136	JUN 137	K6HF 138	KAI1 139	KLK1 140	KLK10 141	KLK11 142	KLK12 143	KLK13 144
KLK14 145	KLK15 146	KLK2 147	KLK3 148	KLK4 149	KLK5 150	KLK6 151	KLK7 152	KLK8 153	KLK9 154	KRT1 155	KRT2A 156
MAP2K4 157	MAP3K1 158	MAPK1 159	MAPK10 160	MAPK11 161	MAPK12 162	MAPK13 163	MAPK14 164	MAPK3 165	MAPK4 166	MAPK6 167	MAPK7 168
MAPK8 169	MAPK9 170	MIB1 171	MMP2 172	MMP9 173	MSMB 174	MTSS1 175	MYC 176	NCOA4 177	NFKB1 178	NFKBIA 179	NKX3-1 180
NOX5 181	NR0B1 182	NR0B2 183	NR1D1 184	NR1D2 185	NR1H2 186	NR1H3 187	NR1H4 188	NR1I2 189	NR1I3 190	NR2C1 191	NR2C2 192
NR2E1 193	NR2E3 194	NR2F1 195	NR2F2 196	NR2F6 197	NR3C1 198	NR3C2 199	NR4A1 200	NR4A2 201	NR4A3 202	NR5A1 203	NR5A2 204
NR6A1 205	NTN4 206	ODZ1 207	PALM2-AKAP2 208	PAP 209	PART1 210	PATE 211	PAWR 212	PCA3 213	PCNA 214	PGR 215	PIAS1 216
PIAS2 217	PIK3CG 218	PLAU 219	PLG 220	PPID 221	TMEM37 222	PRKCA 223	PRKCB1 224	PRKCD 225	PRKCE 226	PRKCG 227	PRKCH 228
PRKCI 229	PRKD3 230	PRKCQ 231	PRKCZ 232	PRKD1 233	PRKD2 234	PRL 235	PSAP 236	PSCA 237	PTEN 238	RARB 239	RASSF1 240
RB1 241	RNASEL 242	RNF14 243	ROBO2 244	SERPINA3 245	SHBG 246	SLC2A2 247	SLC33A1 248	SLC43A1 249	SOX2 250	SRC 251	SRD5A2 252
HSPCB 253	STEAP 254	STEAP2 255	TGFA 256	TGFB1 257	TGFB1I1 258	TGFB2 259	TGFB3 260	TIMP3 261	TNF 262	Pol1 263	PUC18 264
B2M 265	Blank 266	Blank 267	TNFSF10 268	TP53 269	TPM1 270	TPM2 271	18SrRNA 272	AS1R3 273	AS1R2 274	AS1R1 275	AS1 276
B2M 277	B2M 278	ACTB 279	TRPC6 280	TRPS1 281	TYK2 282	VEGF 283	BAS2C 284	BAS2C 285	BAS2C 286	BAS2C 287	BAS2C 288

Figure 5. Human Prostate Cancer Biomarkers Oligo GEArray® DNA Microarray gene layout

Microarrays are nylon membranes designed to study expression of multiple genes involved in the diagnosis or progression of various diseases. Human Prostate Cancer Biomarker Gene microarrays (SuperArray, Frederick, MD) were used to screen 263 biomarker genes involved in prostate cancer. Genes are arranged on the array in groups according to biological function.

V.C.1. Lutein down-regulates the expression of the KLK14 gene



Control **Lutein 10 µM**

Figure 6. Oligo GEArray® Human Prostate Cancer Biomarker Genes Microarray. A down-regulation in KLK14 gene expression was measured when comparing control (untreated) versus 10 µM lutein treated PC-3 cells.

KLK14, located on position number 145 in the Human Prostate Cancer Biomarker Genes Microarray, has been implicated in tumor growth, invasion and angiogenesis (Borgono et al, 2007). In a study by Yousef et al (2006), researchers found a higher expression of KLK14 in stage III prostate cancer patients as compared to those with stage II cancer. The expression of this gene was down-regulated by an average of 84% when comparing control (untreated) to lutein 10 µM-treated PC-3 cells.

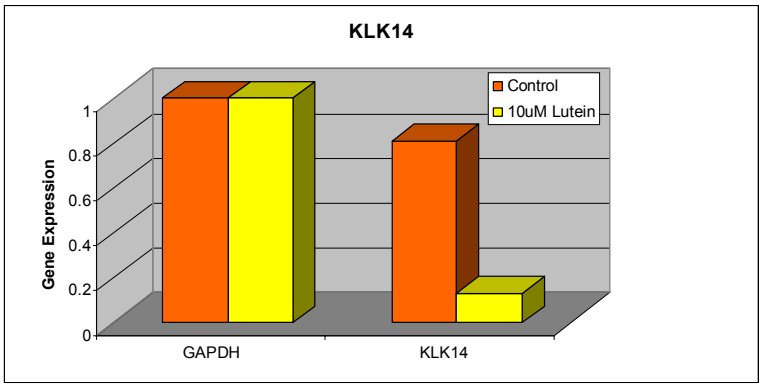
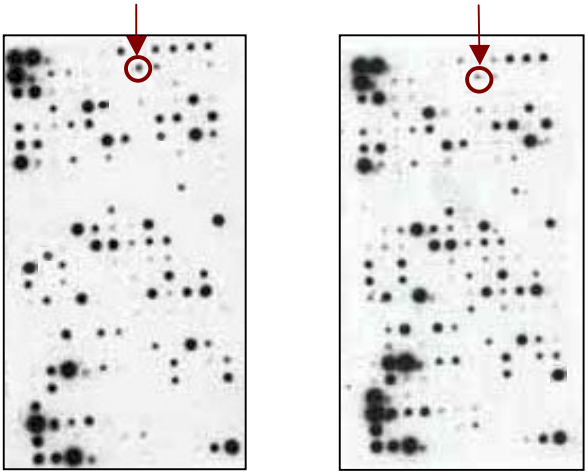


Figure 6a. Lutein down-regulates the expression of KLK14 gene. The expression of GAPDH remains constant while the expression of KLK14 decreases by 84% when comparing the 10 µM lutein treatment to the control.

V.C.2. Lutein down-regulates the expression of the BRCA1 gene



Control **Lutein 10 µM**

Figure 7. Oligo GEArray® Human Prostate Cancer Biomarker Genes Microarray. A down-regulation in BRCA1 gene expression was measured when comparing control (untreated) versus 10 µM lutein treated PC-3 cells.

BRCA1 is located on chromosome 17q21 (Dong, 2006) and can be found on position 20 in the Human Prostate Cancer Biomarker Genes Microarray. Mutated BRCA1 can serve as a marker for aggressive prostate cancer (Mitra et al, 2008) and can also play a role in the increased risk of prostate cancer for men under the age of 65 (Thompson and Easton, 2002). The expression of BRCA1 was down-regulated by an average of 69% when comparing control (untreated) to lutein 10 µM treated PC-3 cells.

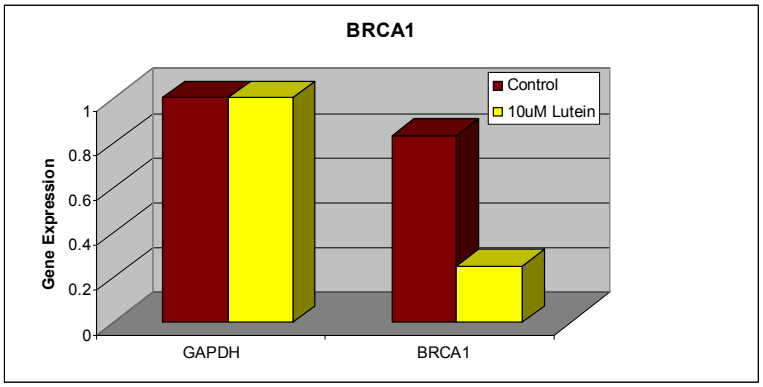
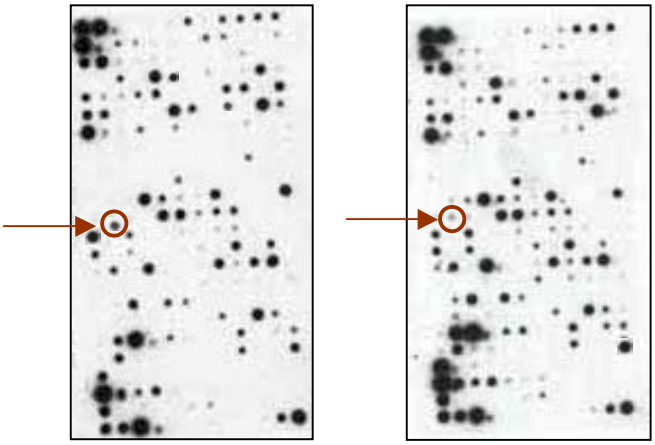


Figure 7a. Lutein down-regulates the expression of **BRCA1** gene. The expression of GAPDH remains constant while the expression of BRCA1 gene decreases by 69% when comparing the 10 µM lutein treatment to the control.

V.C.3. Lutein down-regulates the expression of the IGF1R gene



Control **Lutein 10 µM**

Figure 8. Oligo GEArray® Human Prostate Cancer Biomarker Genes Microarray. A down-regulation in IGF1R gene expression was measured when comparing control (untreated) versus 10 µM lutein treated PC-3 cells.

The IGF1R gene is located on position 122 in the Human Prostate Cancer Biomarker Genes Microarray and is known to mediate tumor cell growth, adhesion and inhibit apoptosis (Hellawell et al, 2002) as well play a role malignant transformation, cell proliferation and tumor survival (Cohen et al, 2004). The expression of IGF1R was down-regulated by an average of 51% when comparing control (untreated) to lutein 10 µM treated PC-3 cells.

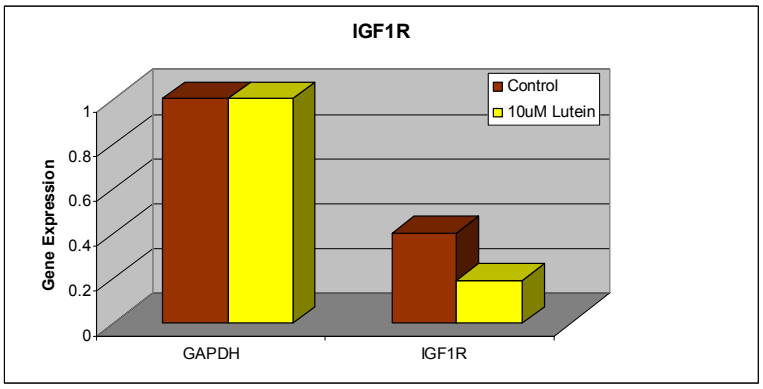
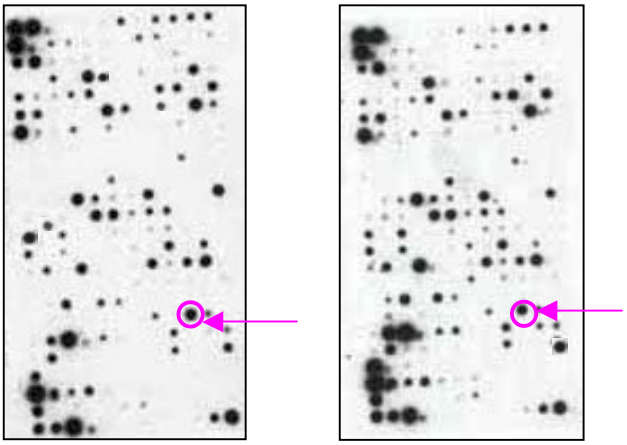


Figure 8a. Lutein down-regulates expression of **IGF1R** gene. The expression of GAPDH remains constant while the expression of IGF1R gene decreases by 51% when comparing the 10 µM lutein treatment to the control.

V.C.4. Lutein down-regulates the expression of the PCA3 gene



Control **Lutein 10 µM**

Figure 9. Oligo GEArray® Human Prostate Cancer Biomarker Genes Microarray. A down-regulation in PCA3 gene expression was measured when comparing control (untreated) versus 10 µM lutein treated PC-3 cells.

PCA3, located on position 213 of the Human Prostate Cancer Biomarker Genes Microarray, has been studied for its potential use as a diagnostic tool in prostate cancer with the probability of a positive repeat biopsy increasing with rising PCA3 scores (Haese et al, 2008). PCA3 scores may be indicative of the stage and aggressiveness of prostate cancer and can also be associated with Gleason scores (Haese et al, 2008). The expression of this gene was down-regulated by an average of 50% when comparing control (untreated) to lutein 10 µM-treated PC-3 cells.

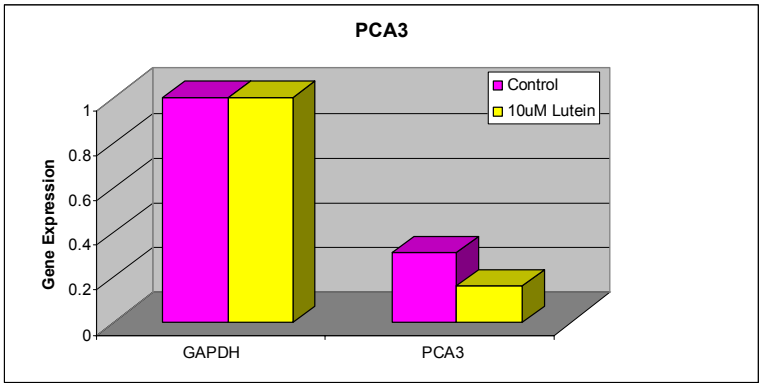
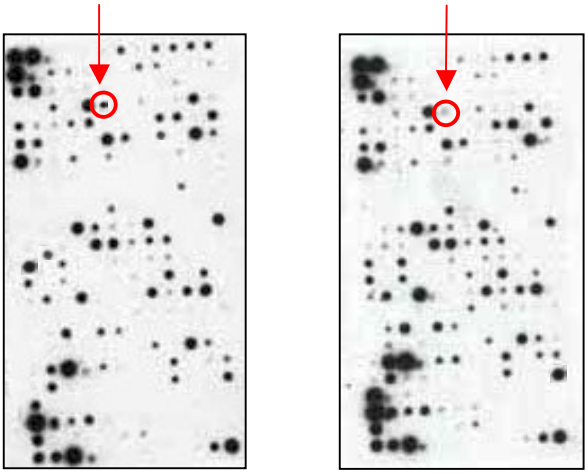


Figure 9a. Lutein down-regulates the expression of PCA3 gene. The expression of GAPDH remains constant while the expression of PCA3 decreases by 50% when comparing the 10 µM lutein treatment to the control.

V.C.5. Lutein down-regulates the expression of the CDK5 gene



Control **Lutein 10 µM**

Figure 10. Oligo GEArray® Human Prostate Cancer Biomarker Genes Microarray. A down-regulation in CDK5 gene expression was measured when comparing control (untreated) versus 10 µM lutein treated PC-3 cells.

CDK5, located on position 42 of the Human Prostate Cancer Biomarker Genes Microarray, is known to be a regulator of neuronal development and plays an important role in prostate cancer motility and metastasis (Strock et al, 2006). The expression of this gene was down-regulated by an average of 48% when comparing control (untreated) to lutein 10 µM-treated PC-3 cells.

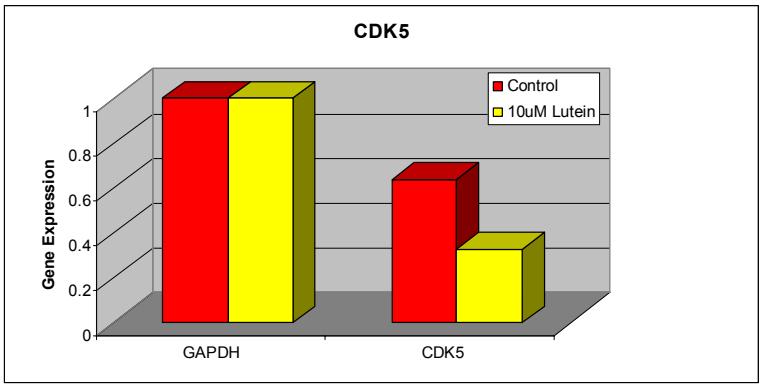
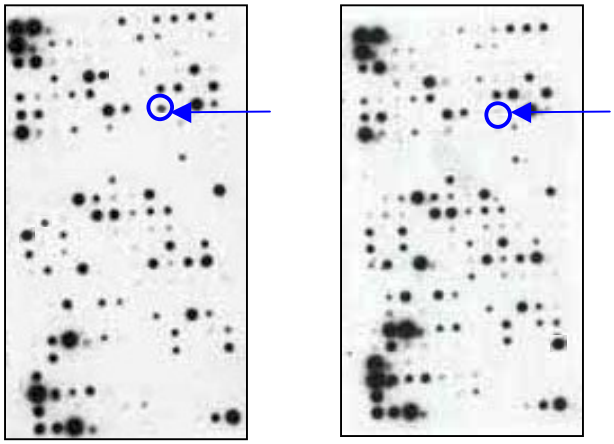


Figure 10a. Lutein down-regulates the expression of **CDK5** gene. The expression of GAPDH remains constant while the expression of CDK5 decreases by 48% when comparing the 10 µM lutein treatment to the control.

V.C.6. Lutein down-regulates the expression of the EGFR gene



Control **Lutein 10 µM**

Figure 11. Oligo GEArray® Human Prostate Cancer Biomarker Genes Microarray. A down-regulation in EGFR gene expression was measured when comparing control (untreated) versus 10 µM lutein treated PC-3 cells.

EGFR, a 170 kDa gene on position 69 on the Human Prostate Cancer Biomarker Genes Microarray, is involved in regulating cell proliferation thereby playing a role in tumor promotion (Zandi et al, 2007). Increased signaling of the EGFR pathway can lead to neoplastic transformations therefore making EGFR a popular target for anti-cancer therapies (Zandi et al, 2007). The expression of this gene was down-regulated by an average of 47% when comparing control (untreated) to lutein 10 µM-treated PC-3 cells.

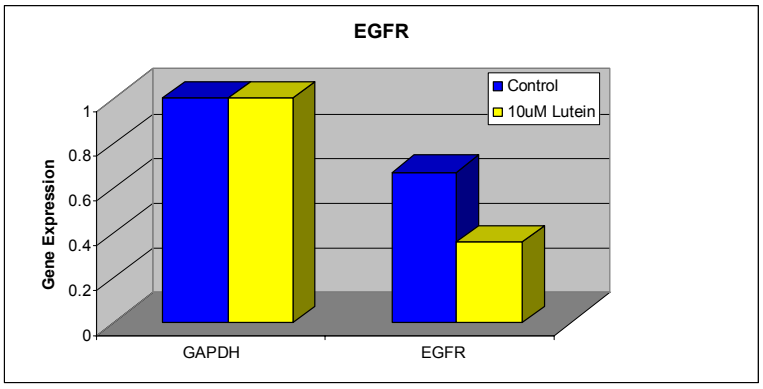
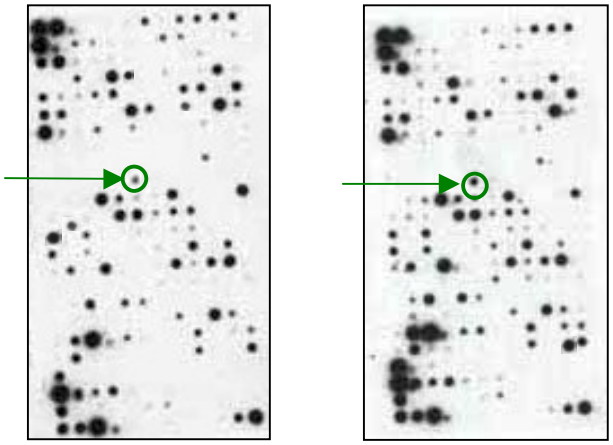


Figure 11a. Lutein down-regulates expression of **EGFR** gene. The expression of GAPDH remains constant while the expression of EGFR gene decreases by 47% when comparing the 10 µM lutein treatment to the control.

V.C.7. Lutein up-regulates the expression of the GSTP1 gene



Control **Lutein 10 µM**

Figure 12. Oligo GEArray® Human Prostate Cancer Biomarker Genes Microarray. An up-regulation in GSTP1 gene expression was measured when comparing control (untreated) versus 10 µM lutein treated PC-3 cells.

GSTP1 is a gene that is located on position 114 of the Human Prostate Cancer Biomarker Genes Microarray. GSTP1 is regarded as a detoxifying enzyme that inactivates electrophilic carcinogens but is often epigenetically inactivated (Meiers et al, 2007). The expression of GSTP1 was up-regulated by an average of 59% when comparing control to 10 µM lutein-treated PC-3 cells.

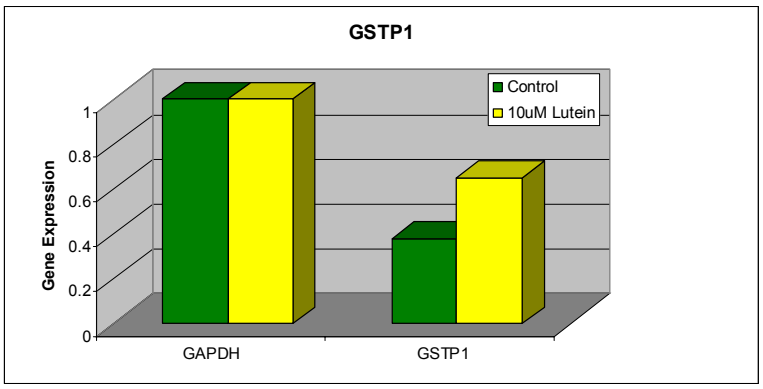
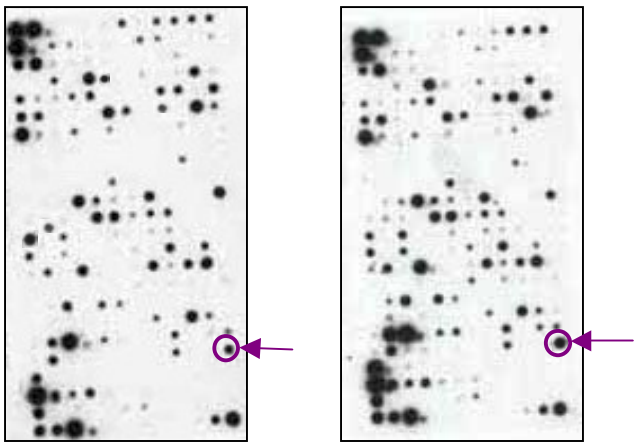


Figure 12a. Lutein up-regulates the expression of **GSTP1** gene. The expression of GAPDH remains constant while the expression of GSTP1 increases by 59% when comparing the 10 µM lutein treatment to the control.

V.C.8. Lutein up-regulates the expression of the RASSF1 gene



Control **Lutein 10 µM**

Figure 13. Oligo GEArray® Human Prostate Cancer Biomarker Genes Microarray. An up-regulation in RASSF1 gene expression was measured when comparing control (untreated) versus 10 µM lutein treated PC-3 cells.

RASSF1 is a tumor suppressor gene and is located on position 240 in the Human Prostate Cancer Biomarker Genes Microarray. Cancer cell lines that express RASSF1 usually display suppressed growth, decreased viability, decreased invasiveness and reduced anchorage however, this gene is inactivated or silenced in a number of tumors (Agathanggelou et al, 2005). The expression of this gene was up-regulated by an average of 35% when comparing control to 10 µM lutein-treated PC-3 cells.

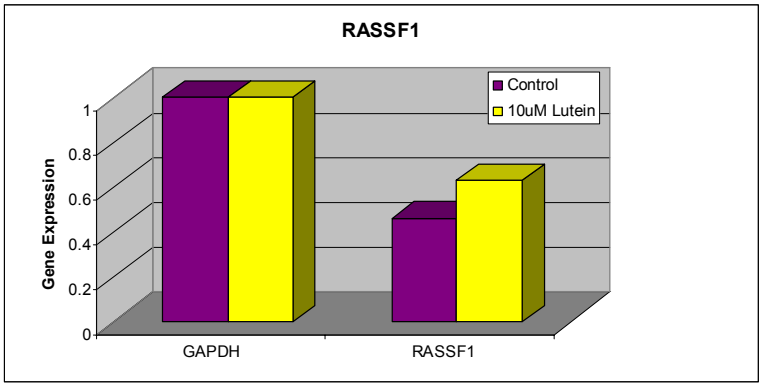


Figure 13a. Lutein up-regulates the expression of **RASSF1** gene. The expression of GAPDH remains constant while the expression of RASSF1 increases by 35% when comparing the 10 µM lutein treatment to the control.

V.D. Real-Time PCR

Real-Time PCR validates modulation of human prostate cancer biomarker genes

Gene	Average Percent Down-regulation
IGF1R	83%
EGFR	60%
BRCA1	50%
CDK5	44%
KLK14	41%
PCA3	40%

Table 5. Average down-regulation of modulated prostate cancer biomarker genes following treatment by 10 μM of lutein

Gene	Average Percent Up-regulation
GSTP1	82%
RASSF1	70%

Table 6. Average up-regulation of modulated prostate cancer biomarker genes following treatment by 10 μM of lutein

Real-time PCR is a sensitive method to validate modulation of gene expression. Experiments are performed in triplicate wells and an average is obtained by utilizing the following calculation:

Average percent modulation =

$$\frac{\text{average control gene expression - average 10 μM lutein gene expression}}{\text{average control gene expression}} \times 100$$

Real-Time PCR quantifies differences in the expression of a specific gene in control and 10 μM lutein samples. Unlike traditional PCR, the amount of product generated is detected as the reactions occur, not at completion. The results are sensitive and precise and are expressed as fold differences in expression levels.

V.D.1. Lutein down-regulates expression of IGF1R gene by Real-Time PCR

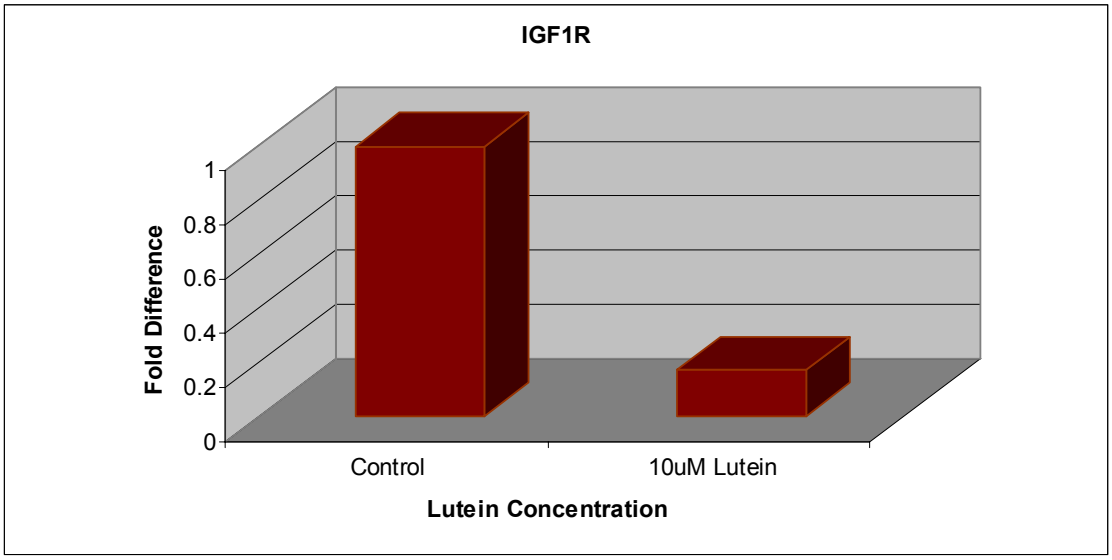


Figure 14. Fold difference in IGF1R gene expression was measured when comparing control (untreated) versus 10 µM lutein treated PC-3 cells.

Real-Time PCR demonstrated an average 0.17 fold difference in IGF1R gene expression when comparing the RNA of control (untreated) versus 10 µM lutein-treated PC-3 cells. The expression of this gene was down-regulated by an average of 83% when comparing control (untreated) to lutein 10 µM-treated PC-3 cells.

V.D.2. Lutein down-regulates expression of EGFR gene by Real-Time PCR

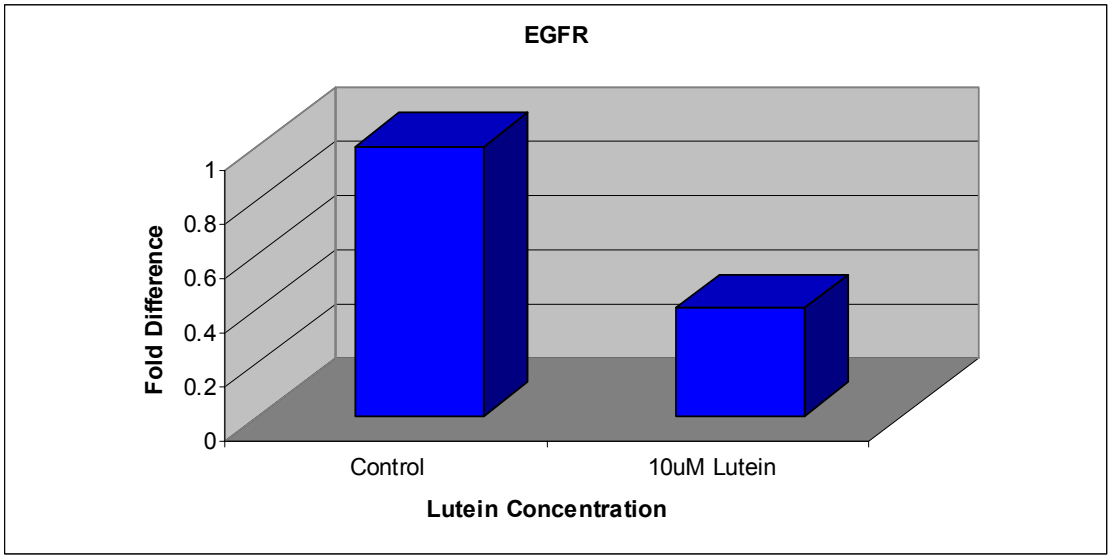


Figure 15. Fold difference in EGFR gene expression was measured when comparing control (untreated) versus 10 µM lutein treated PC-3 cells.

Real-Time PCR demonstrated an average 0.40 fold difference in EGFR gene expression when comparing the RNA of control (untreated) versus 10 µM lutein-treated PC-3cells. The expression of this gene was down-regulated by an average of 60% when comparing control (untreated) to lutein 10 µM-treated PC-3 cells.

V.D.3. Lutein down-regulates expression of BRCA1 gene by Real-Time PCR

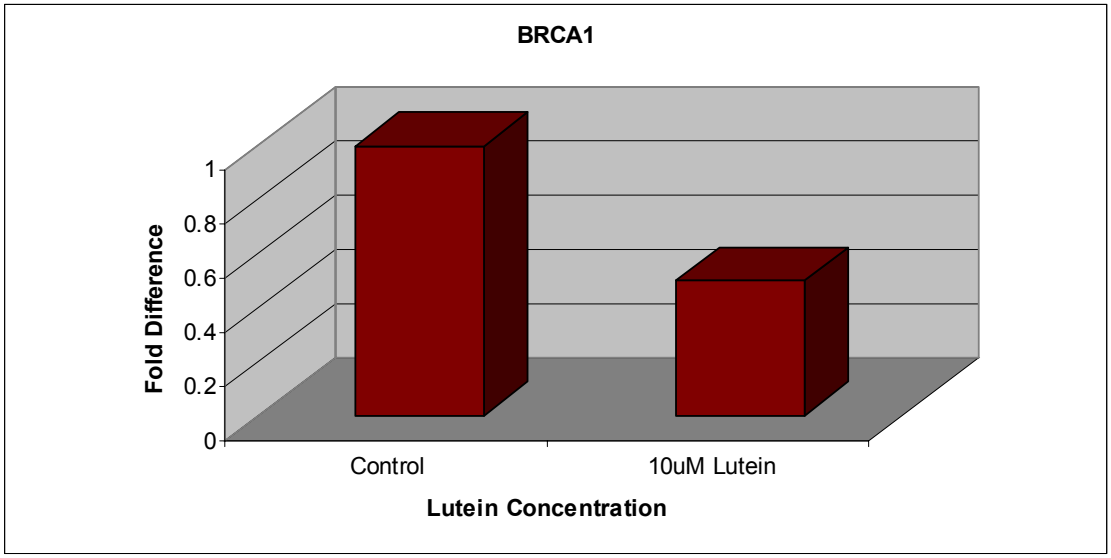


Figure 16. Fold difference in BRCA1 gene expression was measured when comparing control (untreated) versus 10 µM lutein treated PC-3 cells.

Real-Time PCR demonstrated an average 0.51 fold difference in BRCA1 gene expression when comparing the RNA of control (untreated) versus 10 µM lutein-treated PC-3 cells. The expression of this gene was down-regulated by an average of 50% when comparing control (untreated) to lutein 10 µM-treated PC-3 cells.

V.D.4. Lutein down-regulates expression of CDK5 gene by Real-Time PCR

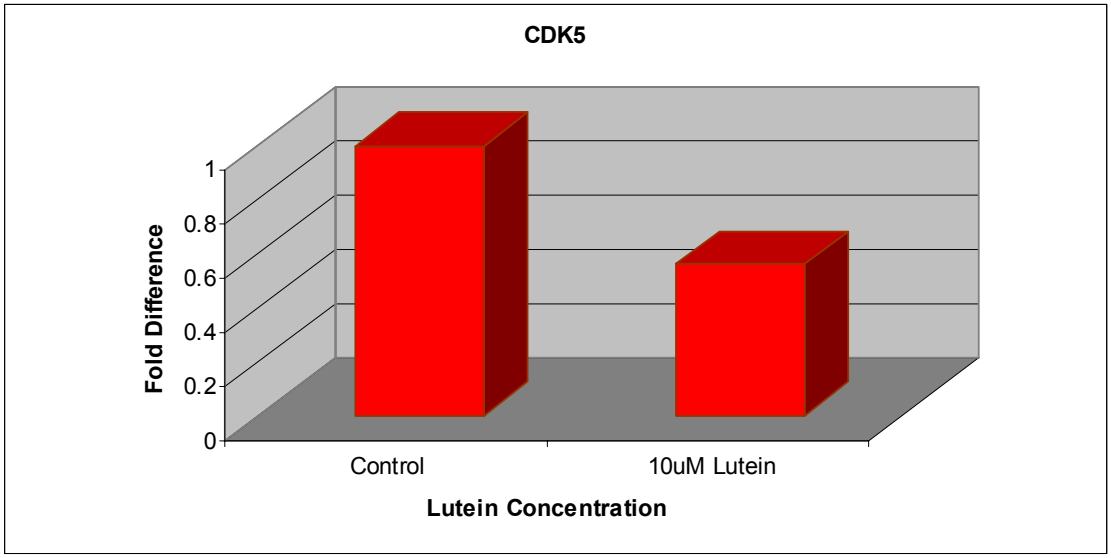


Figure 17. Fold difference in CDK5 gene expression was measured when comparing control (untreated) versus 10 µM lutein treated PC-3 cells.

Real-Time PCR demonstrated an average 0.56 fold difference in CDK5 gene expression when comparing the RNA of control (untreated) versus 10 µM lutein-treated PC-3 cells. The expression of this gene was down-regulated by an average of 44% when comparing control (untreated) to lutein 10 µM-treated PC-3 cells.

V.D.5. Lutein down-regulates expression of KLK14 gene by Real-Time PCR

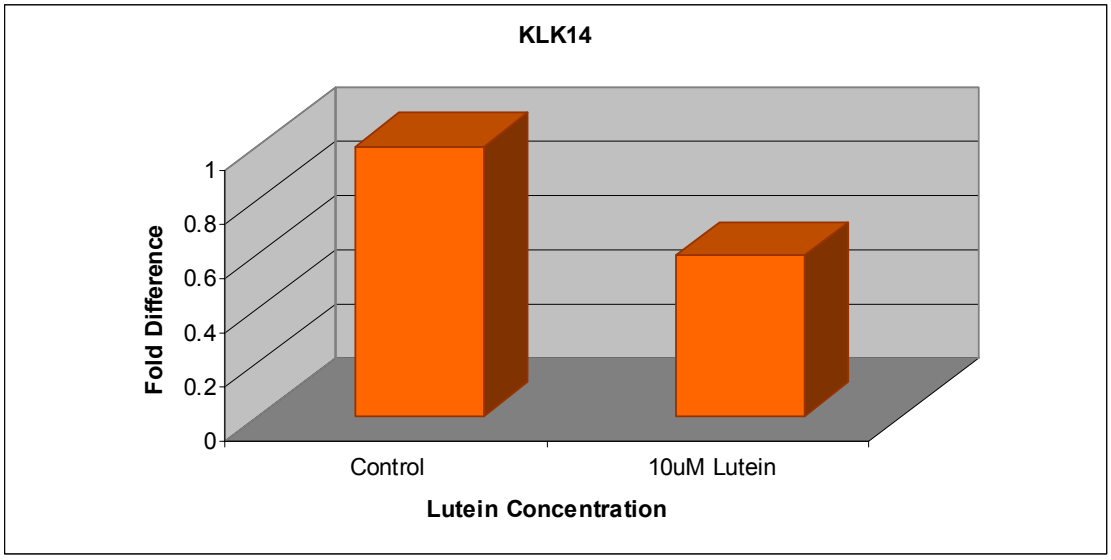


Figure 18. Fold difference in KLK14 gene expression was measured when comparing control (untreated) versus 10 µM lutein treated PC-3 cells.

Real-Time PCR demonstrated an average 0.59 fold difference in KLK14 gene expression when comparing the RNA of control (untreated) versus 10 µM lutein-treated PC-3 cells. The expression of this gene was down-regulated by an average of 41% when comparing control (untreated) to lutein 10 µM-treated PC-3 cells.

V.D.6. Lutein down-regulates expression of PCA3 gene by Real-Time PCR

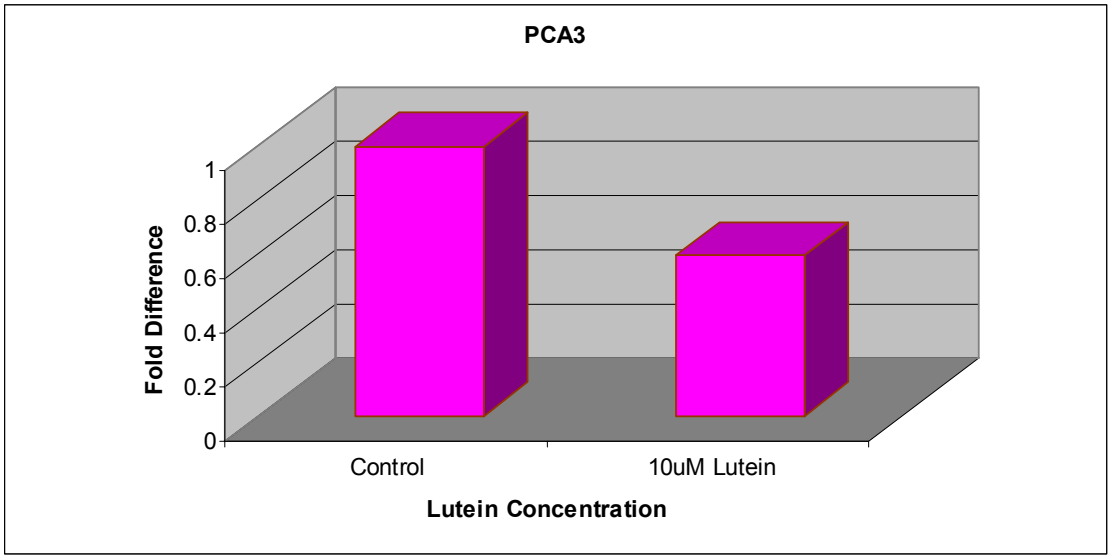


Figure 19. Fold difference in PCA3 gene expression was measured when comparing control (untreated) versus 10 µM lutein treated PC-3 cells.

Real-Time PCR demonstrated an average 0.60 fold difference in PCA3 gene expression when comparing the RNA of control (untreated) versus 10 µM lutein-treated PC-3 cells. The expression of this gene was down-regulated by an average of 40% when comparing control (untreated) to lutein 10 µM-treated PC-3 cells.

V.D.7. Lutein up-regulates expression of GSTP1 gene by Real-Time PCR

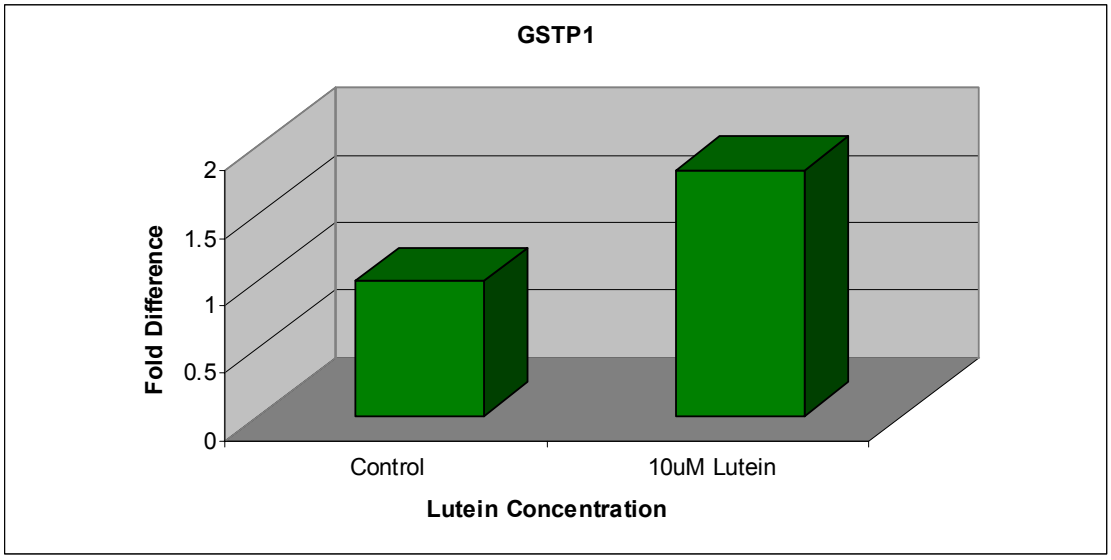


Figure 20. Fold difference in GSTP1 gene expression was measured when comparing control (untreated) versus 10 µM lutein treated PC-3 cells.

Real-Time PCR demonstrated an average 0.83 fold difference in GSTP1 gene expression when comparing the RNA of control (untreated) versus 10 µM lutein-treated PC-3 cells. The expression of this gene was up-regulated by an average of 82% when comparing control (untreated) to lutein 10 µM-treated PC-3 cells.

V.D.8. Lutein up-regulates expression of RASSF1 gene by Real-Time PCR

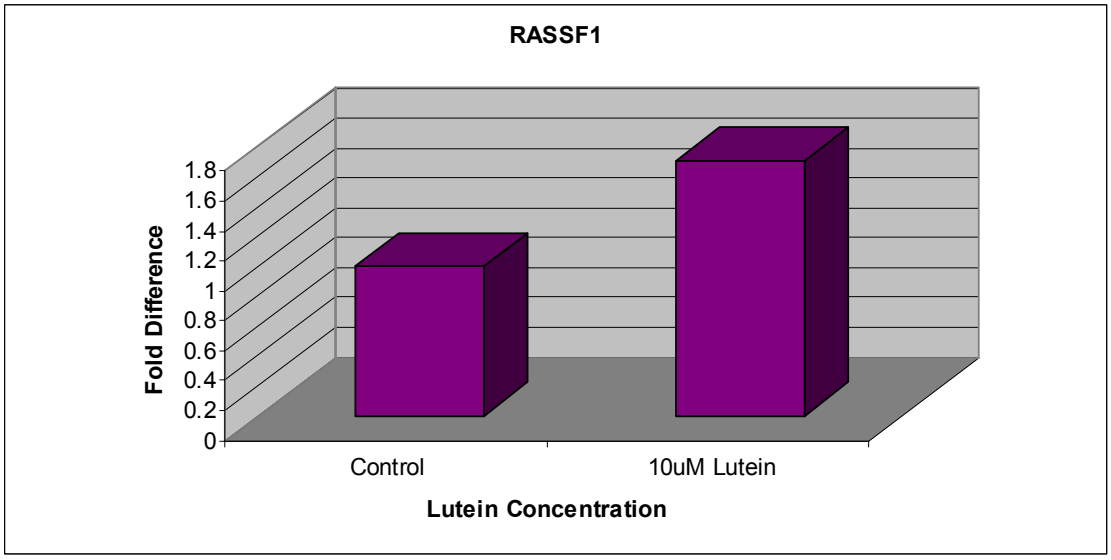


Figure 21. Fold difference in RASSF1 gene expression was measured when comparing control (untreated) versus 10 μM lutein treated PC-3 cells.

Real-Time PCR demonstrated an average 0.71 fold difference in RASSF1 gene expression when comparing the RNA of control (untreated) versus 10 μM lutein-treated PC-3 cells. The expression of this gene was up-regulated by an average of 70% when comparing control (untreated) to lutein 10 μM-treated PC-3 cells.

V.E. Western Blot

Due to a consistent modulation in gene expression from both microarray and Real-Time PCR experiments, in order to further investigate the decreased expression of IGF1R and EGFR and the increased expression of GSTP1 at the protein level, the effect of lutein (10 μM) on IGF1R, EGFR and GSTP1 protein expression was studied by Immunoblot. The intensity of proteins were analyzed using BioRad Quantity One 1D Analysis software. Lutein (10 μM) decreased the protein expression of IGF1R and EGFR while the protein expression of GSTP1 increased. The protein expression of housekeeping β-actin showed little or no change, while the protein expression of IGF1R and EGFR decreased by 40.4% and 33.1% and expression of GSTP1 increased by 30% when comparing control (untreated) to 10 μM lutein treated PC-3 cells.

V.E.1. Lutein decreases the expression of IGF1R protein expression using Western blot

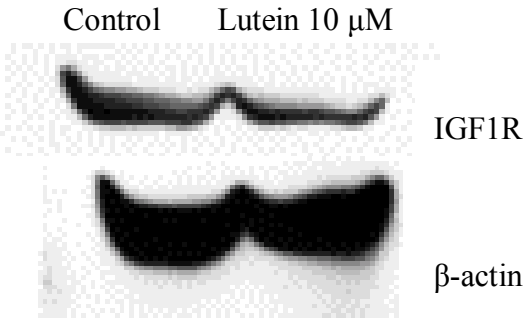


Figure 22. A down-regulation in IGF1R protein expression was measured when comparing control versus 10 μM lutein treatment. The expression of β-actin, a housekeeping gene, remains unchanged.

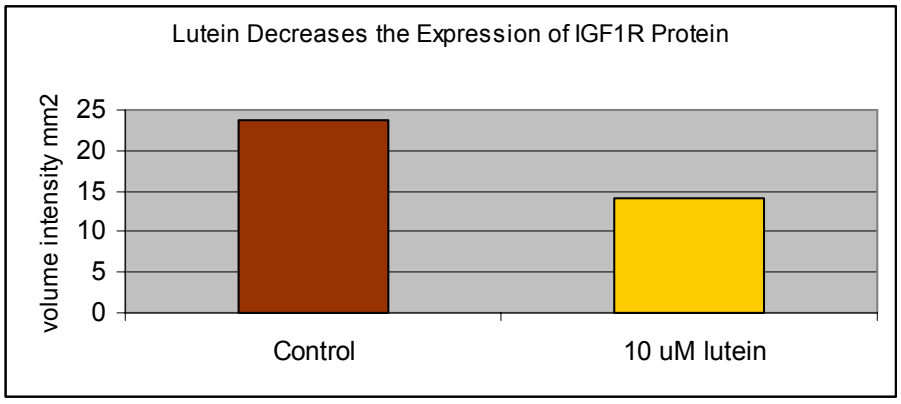


Figure 22a. Lutein down-regulates the protein expression of IGF1R by 40.4% when comparing control to 10 μM lutein treatment.

V.E.2. Lutein decreases the expression of EGFR protein expression using Western blot

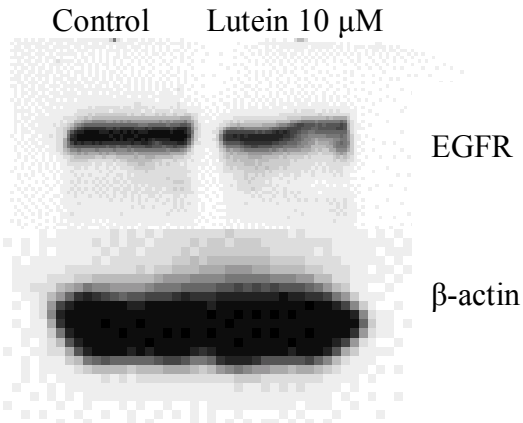


Figure 23. A down-regulation in EGFR protein expression was measured when comparing control versus 10 μM lutein treatment. The expression of β-actin, a housekeeping gene, remains unchanged.

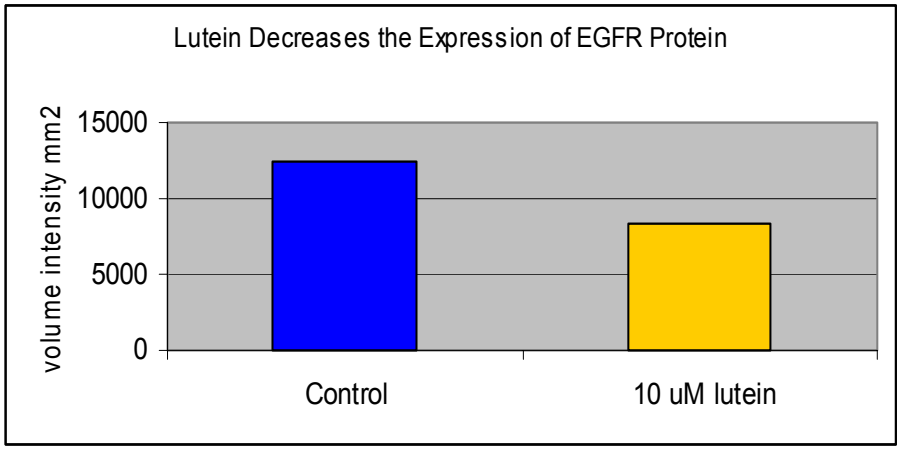


Figure 23a. Lutein down-regulates the protein expression of EGFR by 33.1% when comparing control to 10 μM lutein treatment.

V.E.3. Lutein increases the expression of GSTP1 protein expression using Western blot

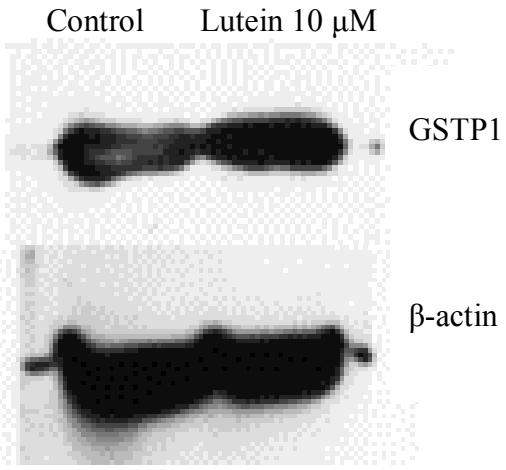


Figure 24. An up-regulation in GSTP1 protein expression was measured when comparing control versus 10 μM lutein treatment. The expression of β-actin, a housekeeping gene, remains unchanged.

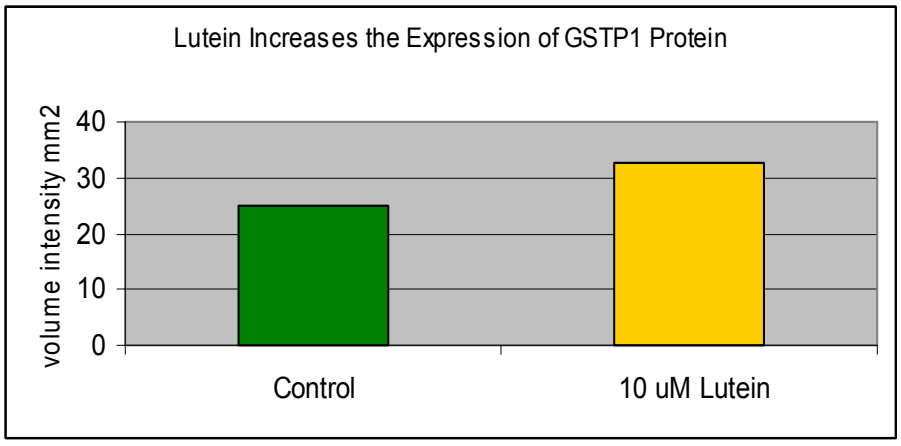


Figure 24a. Lutein up-regulates the protein expression of GSTP1 by 30% when comparing control to 10 μM lutein treatment.

Chapter VI

Discussion

Prostate cancer is the second leading cause of cancer related death in men and will be responsible for an estimated 186,320 new cases of cancer and 28,660 deaths in 2008 (American Cancer Society, 2008). Prostate cancer is the most commonly diagnosed cancer and risk factors generally increase with age, family history, race, overall health and lifestyle (American Cancer Society, 2008).

Prostate tumors, once confirmed by PSA, DRE and biopsy, are assigned a stage and Gleason score to better assess treatment options. In its early stages, prostate cancer is most often treated through surgical removal of the tumor, radiation and some hormonal therapy while more advanced stages of cancer are treated by chemotherapy and hormonal therapy (Garnick and Fair, 1998). If a relapse occurs after treatments, a more aggressive and advanced stage of prostate cancer arises, known as androgen-independent or hormone-refractory prostate cancer and can lead to metastasis.

Researchers have been studying the possible health benefits of various food components in an attempt to find alternative therapies for prevention and treatment of prostate cancer (Adhami et al, 2003; Clinton and Giovannucci, 1998; Lu et al, 2005). Several of these studies have involved the use of nutraceuticals in common foods including curcumin in turmeric, genistein in soy and epigallocathechin-3-gallate (EGCG) in green tea as potential agents against prostate cancer (Mukhopadhyay et al, 2002; Raffoul et al, 2007; Adhami et al, 2003). Lycopene is another nutraceutical that has been researched

extensively for its role against prostate cancer. For example, researchers found a statistically significant inverse association between higher plasma lycopene concentrations and a lower risk of prostate cancer in men who were 65 years or older with no family history of prostate cancer (Wu, Erdman Jr. et al, 2004). Another study suggested the role of lycopene against prostate cancer to include significant down-regulation of EGFR on RNA and protein levels as well as a down-regulation in IGF1R, BRCA1, CDK9, TGFβ2, CDK7 and BCL2 gene expression (Reyes, 2007).

Carotenoids have been the focus of numerous studies and have been implicated for their roles against HIV, cataract, age-related macular degeneration, cardiovascular disease and cancer (Rao and Rao, 2007; Ribaya-Mercado and Blumberg, 2004; Martin and Meydani, 2000; Krinsky and Johnson, 2005). One review specifically focused on xanthophylls which have been shown to modulate apoptosis, inhibit angiogenesis, enhance gap junctional intracellular communication, modulate the immune system and prevent oxidative damage (Ribaya-Mercado and Blumberg, 2004). *In vitro* studies have suggested that lipid soluble bioactive substances in avocados including lutein, synergistically induces cell cycle arrest in PC-3 cells (Lu et al, 2005). Other *in vitro* studies have supported the notion that lutein may act as an anti-inflammatory agent. For example, findings suggested the anti-inflammatory properties of lutein as demonstrated by the decrease in iNOS in mouse macrophage cells (Rafi et al, 2007). *In vivo* studies have demonstrated similar anti-inflammatory and immunosuppressant properties in mice, showing a reduction in reactive oxygen species (ROS) following lutein consumption (Lee et al, 2004). Another *in vivo* study has demonstrated the inhibition of AT3 rat prostate

carcinoma cells by treatment with lutein (Gunasekera et al, 2007). Epidemiological studies have further supported these studies by correlating a lower incidence of prostate cancer with high cruciferous vegetable and fruit intakes (Cohen et al, 2000; McCann et al, 2005).

The objective of the current study was to determine if lutein plays a role in modulating prostate cancer biomarker genes involved in cell differentiation, proliferation, angiogenesis, cell cycle regulation and apoptosis in androgen-independent human prostate cancer PC-3 cell lines. Modulation of these biomarker genes by lutein could indicate a possible role in decreasing the risk, delaying onset or preventing the progression of prostate cancer.

In the current study, a MTT cell viability assay was conducted to determine the highest non-toxic dose of lutein to be used in future experimentation with the PC-3 cell line. Lutein concentrations ranging from 2.5 μM to 50 μM was tested and the results demonstrated that the highest non-toxic dose was 10 μM. PC-3 cells were then cultured, treated with10 μM lutein and RNA and protein were isolated and tested using a spectrophotometer and ethidium bromide agarose gel to obtain an ideal quality. Following the isolation of RNA from untreated and lutein treated PC-3 cells, microarray experiments were conducted using Oligo GEArray® Human Prostate Cancer Biomarker Genes Microarray which is a nylon membrane consisting of 263 genes associated with prognosis, diagnosis, cell proliferation, differentiation, angiogenesis and apoptosis in prostate cancer. Microarrays were conducted in triplicates and results showed up-

regulation of RASSF1 and GSTP1 and a consistent down-regulation of EGFR, IGF1R, BRCA1, CDK5, KLK14 and PCA3.

Epidermal growth factor receptor (EGFR) has been implicated in the progression of prostate cancer to an aggressive, androgen-independent prostate cancer and has been associated with cell cycle progression, inhibition of apoptosis, angiogenesis and tumor cell motility (DiLorenzo et al, 2002; Herbst and Shin, 2001). In the current study, EGFR expression decreased by an average of 47% following lutein treatment. IGF1R, whose expression was consistently down-regulated by an average of 51%, is another gene associated with metastatic cancer which is indicated by the overexpression of IGF1R in malignant versus benign prostate epithelium (Hellawell et al, 2002). BRCA1, with an average 69% decrease, has a hereditary link to breast and prostate cancer (Dong, 2006). Overexpression of mutated BRCA1 represents an increasingly aggressive form of prostate cancer as evident in carriers of these mutations who generally have higher Gleason scores than non-carriers (Mitra et al, 2008). Studies show KLK14 has been implicated in tumor growth, invasion and angiogenesis and increased expression has been demonstrated in stage III tumors as compared to stage I and II tumors (Borgono et al, 2007; Yousef et al, 2003). Our current study shows an average down- regulation of 84% for KLK14. Another gene of recent interest has been PCA3 which researchers have found to be a good indicator of a positive biopsy because of its high specificity to prostate cancer and correlation with tumor volumes and Gleason scores (Haese et al, 2008; Marks et al, 2007; Whitman et al, 2008). Our results have shown an average down-regulation of 50% in PCA3 gene expression for PC-3 cells treated with lutein. CDK5, a gene that

decreased in expression by an average of 48% after lutein treatment, is also a gene of interest due to its involvement in prostate cancer cell motility and metastasis (Strock et al, 2006). RASSF1 and GSTP1 were up-regulated in our current study by 35% and 59% following lutein treatment. RASSF1 is a gene that is silenced in prostate cancer but generally acts to inhibit tumor growth, induce cell cycle arrest and reduces colony formation (Agathanggelou et al, 2005; Li et al, 2005). GSTP1, on the other hand, is the most abundant GST in the human prostate tissue and is involved in the metabolism of a wide range of carcinogenic chemicals (Meiers et al, 2007). Even though GSTP1 has the ability to protect against the progression of prostate cancer, its expression is silenced in most cases (Meiers et al, 2007).

Results from microarray experiments served as a preliminary screening of numerous genes involved in prostate cancer, however Real-Time PCR was conducted to provide validation through more sensitive, accurate and reliable methods for EGFR, IGF1R, BRCA1, CDK5, KLK14, PCA3, GSTP1 and RASSF1. Real-Time PCR experiments were performed in triplicate wells and an average modulation was obtained. GSTP1 and RASSF1 were up-regulated by an average of 82% and 70%, while IGF1R, EGFR, BRCA1, CDK5, KLK14 and PCA3 were down-regulated by an average of 83%, 60%, 50%, 44%, 41% and 40% respectively.

Since the most significant modulation was observed in IGF1R, EGFR and GSTP1, our final objective was to determine protein expression after 10 μM lutein treatment using Western Blot. Western Blot studies indicated a 40.4% decrease in IGF1R expression,

33.1% decrease in EGFR expression and a 30% increase in GSTP1 expression following treatment with lutein. Results from microarrays, Real-Time PCR and Western Blot indicate the potential chemopreventative effects of lutein on EGFR and IGF1R which are involved in the progression of prostate cancer to a more aggressive and metastatic disease as well as the effect of lutein on GSTP1 which is a tumor suppressor gene.

Elevated expression of EGFR, a growth factor receptor tyrosine kinase, has been implicated in promoting solid tumor growth (Nicholson et al, 2001). The activation of EGFR signaling pathway is responsible for inhibition of apoptosis, progression to angiogenesis, cellular adhesion, differentiation and cell growth (Rocha-Lima et al, 2007) and is therefore a target for anti-cancer therapy. EGFR expression is correlated with clinical outcome for patients with head and neck, ovarian, cervical, bladder and esophageal cancers (Rocha-Lima et al, 2007) and overexpression is also observed in breast, prostate, renal, colon, ovarian, bladder and pancreatic tumors (Rocha-Lima et al, 2007; Herbst and Shin 2002; DiLorenzo et al, 2002).

Currently, the most clinically effective and advanced strategies for EGFR inhibition include the use of monoclonal antibody blockades which involve extracellular binding to EGFR and tyrosine kinase inhibitors which bind intracellularly to EGFR tyrosine kinase (Rocha-Lima et al 2007; Herbst and Shin, 2001). Monoclonal antibodies attach to EGFR and hinder the attachment and activation of the receptor leading to an anti-proliferative effect (Zandi et al, 2007). The most extensively studied antibody of this kind is Cetuximab (Erbitux™) which is a human-mouse monoclonal antibody that has recently

been approved by the Food and Drug Administration for patients with advanced colorectal cancer (Rocha-Lima et al, 2007; Zandi et al, 2007; FDA, 2004). It has been shown to prevent ligand binding and induce receptor degradation which leads to the inhibition of cancer cell growth by blocking G1 phase, inducing cell cycle arrest, promoting apoptosis and inhibiting tumor angiogenesis (Rocha-Lima et al, 2007). Studies have also implied that Cetuximab could make cancer cells more sensitive to chemotherapy and radiation (Rocha-Lima et al, 2007), however there are many side effects to this drug that need to be considered. Some side effects include rash, fatigue, fever, constipation, abdominal pain and infrequent interstitial lung disease but it is difficult to determine the exact cause of these effects since so many factors can be involved (FDA, 2004).

Other monoclonal antibodies that are currently undergoing clinical trials as EGFR inhibitors include panitumumab (ABX-EGF), matuzumab (EMD-72000), nimutozumab (h-R3), MDX-447 and mAb806 (Rocha-Lima et al, 2007). Panitumumab, which is currently in phase II/III trials, competitively inhibits EGFR ligand binding, promotes receptor internalization and prevents tyrosine kinase phosphorylation, similar to the functions of cetuximab however one distinction between these two drugs is that panitumumab does not stimulate receptor degradation (Rocha-Lima et al, 2007).

Overexpression of EGFR has been correlated with decreased survival in cancers including head and neck, bladder, ovarian, cervical, esophageal cancers and has been implicated in the progression to an androgen-independent prostate cancer (Nicholson et

al, 2001; DiLorenzo et al, 2002). To inhibit the activity of EGFR, it is important to hinder or disrupt receptor signaling activity by using tyrosine kinase inhibitors (Zandi et al, 2007). Two EGFR specific tyrosine kinase inhibitors, Gefitinib (ZD1839, Iressa™) and Erlotinib (Tarceva™), have been approved for the treatment of patients with specific advanced or metastatic cancers although the approval of Gefitinib is under review again after two failed FDA trials and Erlotinib is approved for use only after failure of at least one prior chemotherapy procedure (Zandi et al, 2007; FDA, 2008). Nevertheless, both inhibitors are thought to selectively prevent phosphorylation of EGFR while inhibiting tumor growth, angiogenesis and promotion of apoptosis (Zandi et al, 2007; Rocha-Lima et al, 2007). Although there are numerous methods for targeting EGFR, the benefits of these treatments or in some cases, the reasons behind treatment failure still remain vague (Zandi et al, 2007).

IGF1R, another growth factor receptor, is known to initiate a cascade of events in cancer leading to the stimulation of cell proliferation, induction of neoplatic transformation, promotion of tumorigenesis with significant over-expression observed in prostate cancer as well as lung, colon and breast cancers (Hellawell et al, 2002; Sachdev and Yee, 2007). A variety of approaches have been considered for anti-cancer therapy against IGF1R including neutralization or reduction of IGF1 levels and finding antibodies against IGF1 using IGF1R inhibitors (Sachdev and Yee, 2007). Another approach includes inhibiting tumor growth by using a Growth Hormone-Releasing Hormone (GHRH) antagonist that regulates hepatic synthesis and secretion of IGF-1 (Sachdev and Yee, 2007). Two examples of GHRH include JV-1-36 which inhibits tumor proliferation in breast cancer

cells and JV-1-38 which is an inhibitor of non-small-cell lung cancer cells in mice (Sachdev and Yee, 2007). Like EGFR, monoclonal antibodies have also been considered to inhibit IGF1R function. An example of this is the murine monoclonal antibody αIR-3 which inhibits the attachment of IGF-1 to human breast cancer cells and restrains the growth of such cells (Cohen et al, 2005). Antibody αIR-3 possesses chemopreventative activity and may be applicable for use in chemotherapy although its clinical use may be limited (Cohen et al, 2005). Another antibody that is being studied as an IGF1R target is CP-751,871, a human monoclonal antibody that binds specifically to IGF1R with high affinity, restricts the binding between IGF1 and its receptor and inhibits the activation of IGF1R which in turn inhibits tumor proliferation (Cohen et al, 2005). Currently, Pfizer is studying the actions of CP-751,871 in phase III trials for use in non-small cell lung cancer and in phase II trials for use in cancers including prostate, breast, colorectal and Ewing's sarcoma (Pfizer, 2008). INSM-18, a tyrosine kinase inhibitor of IGF1R is also currently undergoing clinical trials for prostate cancer under the supervision of Insmed Incorporated and University of California, San Francisco School of Medicine (Insmed Incorporated, 2004). Thus far, INSM-18 has been determined to trigger tumorigenesis although studies are ongoing (Insmed Incorporated, 2004)

Even though researchers are studying the effectiveness of therapies against tumor promoting genes, another possible approach can be to study the activation or amplification of silenced tumor suppressor genes in cancer. For example, one approach could be to study GSTP1, a tumor suppressor gene that is responsible for the detoxification of carcinogenic compounds (Li et al, 2005) and can be studied for its

possible application in anti-cancer therapy. GSTP1 is the most abundant GST present in human prostate tissue but its presence is lacking in 95% sporadic prostate cancers (Cohen et al, 2000). GSTP1 is susceptible to inactivation or silencing by epigenetic alterations which causes increased cell vulnerability to oxidative DNA damage and genetic modulations in cancer (Li et al, 2005). For example, in prostate cancer this inactivation occurs in the beginning stages of carcinogenesis, leaving normal prostate cells vulnerable to carcinogens (Meiers et al, 2007).

Considerable attempts have been made to develop chemopreventative agents that target various growth factors and tumor suppressor genes involved in prostate cancer most of which are undergoing clinical trials with varying levels of success. The lack of immediate and successful intervention causes concern with a disease that is the second leading cause of cancer-related death in men. Another cause for concern is the high specificity of agents currently under review to a specific gene instead of an agent that targets a compilation of the genes that have the most impact on prostate cancer. It is evident, however that currently utilized clinical therapies, agents that are under review in clinical trials and food components like lutein and other nutraceuticals may improve overall treatment either alone or in combination to possible prevent or delay the progression of prostate cancer.

From the current research it can be concluded that dietary lutein has the potential to prevent or delay the advancement of prostate cancer through the possible inhibition of angiogenesis, tumorigenesis, cell differentiation, proliferation or metastasis. Results from the Oligo GEArray® Human Prostate Cancer Biomarker Genes Microarray and Real-

Time PCR indicate that lutein has the potential to modulate several biomarker genes involved in prostate cancer. The effect of lutein on IGF1R, EGFR and GSTP1 protein expression further supports the notion that lutein may be an effective chemopreventative agent against prostate cancer. Several epidemiological studies show significant findings of the inverse relation between lutein consumption and the incidence of prostate cancer while both *in vivo* and *in vitro* studies show the general effect of lutein against inflammation or various cancers although no mechanism of action was proposed. The current study therefore researches the effect of lutein on the expression of IGF1R, EGFR and GSTP1 through the analysis of mRNA and protein expression in hormone-refractory human prostate cancer PC-3 cell lines and suggests the potential of lutein as a therapeutic agent to be used either alone or in conjunction with existing clinical therapies. Further research may be needed to study the combined effects of lutein with chemotherapy, radiation or hormone therapy to determine optimal variations of treatment. Further research on the use of lutein as a potential EGFR or IGF1R inhibitor, GSTP1 gene promoter or as a therapeutic agent against prostate cancer either alone or in conjunction with currently approved therapies, are warranted.

Chapter VII

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Chapter VIII

Appendix

TABLE OF ABBREVIATIONS	
BRCA1	Breast Cancer Gene 1
CDK5	Cyclin Dependant Kinase 5
DRE	Digital Rectal Exam
EGFR	Epidermal Growth Factor Receptor
FDA	Food and Drug Administration
GRAS	Generally Regarded As Safe
GSTP1	Glutathione S-Transferase Pi 1
HDL	High Density Lipoprotein
IGF1R	Insulin-Like Growth Factor 1 Receptor
KLK14	Kallikrein 14
LDL	Low Density Lipoprotein
PCA3	Prostate Cancer Antigen 3
PSA	Prostate Specific Antigen
RASSF1	Ras Association Domain Family 1
Real-Time PCR	Real-Time Polymerase Chain Reaction
USDA	United States Department of Agriculture

APPENDIX 2

Oligo GEArray® Human Prostate Cancer Biomarker Gene Expressions – Set 1

GENES	CONTROL	Lutein 10μM	GENES	CONTROL	Lutein 10μM
RPS27A	0.98	0.96	**CDK5**	0.76	0.3
RPS27A	0.94	0.95	CDK6	0.26	0.1
AGR2	0.63	0.62	CDK7	0.48	0.38
AGTR2	0.2	0.57	CDK8	0.32	0.07
AIG1	0.58	0.58	CDK9	0.38	0.21
AKAP1	0.74	0.34	CDKN1A	2.92	2.2
AKT1	2	0.83	CDKN1B	0.29	0.56
APC	0.31	0.46	CDKN1C	1.49	0.94
APOC1	0.48	0.68	CDKN2A	0.52	0.91
GAPDH	1	1	CDKN2B	0.75	0.57
GAPDH	1	1	CDKN2C	1.13	1.39
GAPDH	1	0.98	CDKN3	2.96	3.47
RPS27A	1.01	0.97	CHGA	0.33	0.19
AR	0.9	0.95	CHGB	0.23	0.12
BAK1	1.25	0.94	CLDN3	0.72	0.35
BAX	0.6	0.59	CLN3	2.23	2.87
BCL2	0.37	0.23	CLU	3.85	4.45
BCL2L1	0.71	0.56	COL1A1	0.27	0.25
BMP6	0.32	0.39	COL6A1	3.22	3.67
BRCA1	1.6	0.51	CYB5A	3.1	2.55
CANT1	1.1	0.5	CYC1	4.67	3.63
CASP1	0.29	0.19	DAB2IP	0.69	0.44
CASP3	0.22	0.12	DAPK1	0.35	0.08
CASP7	0.23	0.27	DES	0.32	0.1
CAV1	3.35	2.23	DYNLL1	4.57	4.25
CCND1	4.5	4.98	E2F1	1.72	2.01
CD164	1.5	0.47	EGF	0.41	0.22
CD44	0.28	0.13	**EGFR**	0.9	0.45
CDH1	0.35	0.15	EGR3	0.2	0.17
CDH10	0.21	0.1	ELAC2	4	4.86
CDH12	0.38	0.18	ELL	0.24	0.46
CDH13	0.12	0.22	ENO1	5.41	5.19
CDH18	0.34	0.13	ENO2	0.78	0.89
CDH19	0.33	0.27	ENO3	0.23	0.33
CDH20	0.12	0.2	ERBB2	1.48	0.84
CDH7	0.11	0.18	MAPK15	0.45	0.09
CDH8	0.19	0.23	ESR1	1.03	0.71
CDH9	0.17	0.3	ESR2	0.55	0.18
CDK2	1.45	1.06	EZH1	0.54	0.48
CDK3	0.17	0.05	EZH2	0.73	0.49
CDK4	4.23	4.44	FASN	0.78	0.21

APPENDIX 2 (Continued):

Oligo GEArray® Human Prostate Cancer Biomarkers Microarray Gene Expression – Set 1

GENES	CONTROL	Lutein 10μM	GENES	CONTROL	Lutein 10μM
FGF1	0.1	0.19	IL12A	0.9	0.98
FGF10	0.12	0.12	IL1A	0.34	0.37
FGF11	0.23	0.12	IL1B	4.44	4.62
FGF12	0.22	0.05	IL2	0.16	0.38
FGF13	0.15	0.36	IL24	0.31	0.32
FGF14	0.21	0.28	IL29	0.28	0.2
FGF16	0.32	0.1	ILK	3.61	3.38
FGF17	0.33	0.39	INHA	0.25	0.37
FGF18	0.61	0.54	INSL3	0.45	0.35
FGF19	0.49	0.51	INSL4	0.35	0.3
FGF2	0.39	0.27	ITGA1	0.15	0.23
FGF20	0.25	0.07	JUN	2.19	3.28
FGF21	0.36	0.13	K6HF	3.13	2.51
FGF22	0.4	0.11	CD82	0.8	0.79
FGF23	0.14	0.16	KLK1	1.98	1.8
FGF3	0.27	0.07	KLK10	0.62	1.03
FGF4	0.17	0.16	KLK11	0.09	0.36
FGF5	0.22	0.11	KLK12	0.27	0.35
FGF6	0.11	0.36	KLK13	0.15	0.42
FGF7	0.23	0.05	**KLK14**	1.84	0.46
FGF8	0.46	0.1	KLK15	0.17	0.04
FGF9	0.19	0.28	KLK2	1.4	2.03
FHIT	0.37	0.21	KLK3	0.31	0.31
ARMC9	0.93	0.85	KLK4	0.38	0.27
FLJ25530	0.2	0.46	KLK5	0.25	0.33
FOLH1	0.13	0.33	KLK6	0.22	0.5
PAGE1	0.15	0.08	KLK7	0.36	0.46
PAGE4	0.25	0.19	KLK8	0.14	0.39
GGT1	0.19	0.15	KLK9	0.15	0.39
GNRH1	0.27	0.29	KRT1	0.12	0.4
GRP	0.5	0.25	KRT2A	0.18	0.33
GSTP1	2.28	3.72	MAP2K4	2.28	2.32
HIF1A	0.28	0.2	MAP3K1	0.49	0.31
HIP1	0.22	0.23	MAPK1	0.83	0.73
HK2	0.39	0.41	MAPK10	0.26	0.18
HK3	0.21	0.43	MAPK11	0.22	0.24
HRAS	0.47	0.35	MAPK12	0.16	0.43
KRT2B	0.15	0.3	MAPK13	0.31	0.49
IGF1	0.09	0.1	MAPK14	0.47	0.39
IGF1R	0.43	0.07	MAPK3	3.25	2.67
IGF2	0.35	0.76	MAPK4	0.25	0.32
IGFBP3	4.55	4.91	MAPK6	2.47	0.88
IGFBP6	1.36	1.76	MAPK7	0.15	0.15

APPENDIX 2 (Continued):

Oligo GEArray® Human Prostate Cancer Biomarkers Microarray Gene Expression - Set 1

GENES	CONTROL	Lutein 10μM	GENES	CONTROL	Lutein 10μM
MAPK8	0.95	0.59	PATE	0.43	0.32
MAPK9	1.45	2.58	PAWR	1.62	0.51
MIB1	0.27	0.1	**PCA3**	0.42	0.15
MMP2	4.02	4.65	PCNA	4.17	4.26
MMP9	0.52	0.37	PGR	0.89	1.17
MSMB	0.29	0.29	PIAS1	0.23	0.43
MTSS1	0.39	0.3	PIAS2	0.31	0.3
MYC	3.2	3.92	PIK3CG	3.22	5.18
NCOA4	0.57	0.76	PLAU	5.02	5.68
NFKB1	2.84	2.59	PLG	0.58	0.62
NFKBIA	4.23	4.84	PPID	1.38	0.83
NKX3-1	0.45	0.32	TMEM37	1.8	1.42
NOX5	0.13	0.19	PRKCA	0.33	0.48
NR0B1	0.16	0.07	PRKCB1	0.35	0.26
NR0B2	0.26	0.07	PRKCD	1.34	2.25
NR1D1	0.24	0.42	PRKCE	0.28	0.13
NR1D2	0.3	0.2	PRKCG	0.83	0.89
NR1H2	0.52	0.31	PRKCH	0.8	0.61
NR1H3	0.46	0.45	PRKCI	0.35	0.22
NR1H4	0.27	0.23	PRKD3	3.02	3.65
NR1I2	0.32	0.57	PRKCQ	0.49	0.49
NR1I3	0.34	0.34	PRKCZ	0.33	0.3
NR2C1	0.36	0.25	PRKD1	0.24	0.21
NR2C2	0.39	0.27	PRKD2	0.22	0.42
NR2E1	0.2	0.2	PRL	0.33	0.43
NR2E3	0.75	0.42	PSAP	0.21	0.26
NR2F1	3.89	3.67	PSCA	1.45	0.82
NR2F2	0.27	0.28	PTEN	0.59	0.15
NR2F6	2.1	1.71	RARB	0.12	0.04
NR3C1	0.57	0.81	**RASSF1**	2.26	3.64
NR3C2	0.26	0.28	RB1	4.87	5.29
NR4A1	0.09	0.43	RNASEL	0.49	0.44
NR4A2	0.48	0.29	RNF14	0.47	0.16
NR4A3	0.5	0.14	ROBO2	0.21	0.08
NR5A1	0.41	0.58	SERPINA3	0.11	0.21
NR5A2	0.11	0.49	SHBG	0.15	0.18
NR6A1	0.15	0.2	SLC2A2	0.23	0.16
NTN4	0.63	0.48	SLC33A1	0.3	0.09
ODZ1	0.61	0.29	SLC43A1	0.17	0.22
PALM2-AKAP2	0.68	0.73	SOX2	0.36	0.07
REG3A	0.59	0.37	SRC	0.14	0.1
PART1	0.41	0.43	SRD5A2	0.12	0.11

APPENDIX 2 (Continued):

Oligo GEArray® Human Prostate Cancer Biomarkers Microarray Gene Expression - Set 1

GENES	CONTROL	Lutein 10μM
HSPCB	5.69	5.47
STEAP1	2.79	3.86
STEAP2	1.68	0.65
TGFA	3.1	2.62
TGFB1	0.36	0.52
TGFB1I1	0.36	0.18
TGFB2	0.32	0.13
TGFB3	0.09	0.21
TIMP3	0.11	0.27
TNF	0.13	0.26
Pol1	0.11	0.23
PUC18	0.14	0.2
B2M	4.58	4.28
Blank	0	0
Blank	0	0
TNFSF10	0.6	0.48
TP53	0.35	0.33
TPM1	0.36	0.12
TPM2	0.57	0.62
18SrRNA	0.29	0.45
AS1R3	0.09	0.32
AS1R2	0.18	0.17
AS1R1	0.29	0.09
AS1	0.24	0.15
B2M	1.2	1
B2M	0.96	0.98
ACTB	5.4	5.71
TRPC6	0.38	0.54
TRPS1	0.52	0.54
TYK2	0.49	0.32
VEGFA	0.41	0.2
BAS2C	0.24	0.21
BAS2C	0.57	0.43
BAS2C	0	0.77
BAS2C	0.23	0
BAS2C	0	0

APPENDIX 3:

Oligo GEArray® Human Prostate Cancer Biomarkers Microarray Gene Expression - Set 2

GENES	CONTROL	Lutein 10μM	GENES	CONTROL	Lutein 10μM
RPS27A	0.93	0.76	CDK6	0.25	0.23
RPS27A	0.87	0.88	CDK7	0.66	0.5
AGR2	0.37	0.41	CDK8	0.27	0.23
AGTR2	0.23	0.22	CDK9	0.34	0.32
AIG1	0.36	0.22	CDKN1A	2.56	2.73
AKAP1	0.36	0.28	CDKN1B	0.29	0.23
AKT1	1.34	0.67	CDKN1C	1.31	1.09
APC	0.24	0.26	CDKN2A	0.76	0.64
APOC1	0.57	0.48	CDKN2B	0.69	0.32
GAPDH	0.9	1	CDKN2C	1.57	1.03
GAPDH	1	0.9	CDKN3	3.15	3.12
GAPDH	1	0.99	CHGA	0.26	0.19
RPS27A	0.96	0.99	CHGB	0.22	0.27
AR	0.81	0.87	CLDN3	0.25	0.23
BAK1	1.41	0.68	CLN3	2.5	2.78
BAX	0.76	0.59	CLU	3.28	4.76
BCL2	0.33	0.26	COL1A1	0.48	0.3
BCL2L1	0.23	0.26	COL6A1	2.99	3.45
BMP6	0.23	0.15	CYB5A	3.11	2.4
BRCA1	1.73	0.65	CYC1	4.91	3.48
CANT1	1.24	0.41	DAB2IP	0.33	0.4
CASP1	0.29	0.21	DAPK1	0.25	0.2
CASP3	0.23	0.18	DES	0.25	0.2
CASP7	0.32	0.27	DYNLL1	4.71	4.39
CAV1	3.9	2.11	E2F1	1.93	2.06
CCND1	5.34	4.59	EGF	0.23	0.22
CD164	1.47	0.48	**EGFR**	0.4	0.29
CD44	0.3	0.39	EGR3	0.25	0.23
CDH1	0.36	0.28	ELAC2	4.65	4.25
CDH10	0.27	0.18	ELL	0.38	0.37
CDH12	0.25	0.18	ENO1	5.03	5.05
CDH13	0.28	0.25	ENO2	0.64	0.84
CDH18	0.4	0.23	ENO3	0.46	0.31
CDH19	0.49	0.32	ERBB2	1.61	1.06
CDH20	0.35	0.16	MAPK15	0.31	0.22
CDH7	0.27	0.15	ESR1	0.9	0.86
CDH8	0.22	0.22	ESR2	0.28	0.2
CDH9	0.39	0.27	EZH1	0.26	0.22
CDK2	1.81	0.76	EZH2	0.26	0.38
CDK3	0.32	0.26	FASN	0.61	0.7
CDK4	4.42	4.1	FGF1	0.33	0.23
CDK5	0.51	0.5	FGF10	0.27	0.25

APPENDIX 3 (Continued):

Oligo GEArray® Human Prostate Cancer Biomarkers Microarray Gene Expression - Set 2

GENES	CONTROL	Lutein 10μM	GENES	CONTROL	Lutein 10μM
FGF11	0.27	0.28	IL12A	0.56	0.87
FGF12	0.28	0.25	IL1A	0.29	0.27
FGF13	0.3	0.25	IL1B	4.67	4.56
FGF14	0.34	0.25	IL2	0.19	0.3
FGF16	0.23	0.22	IL24	0.28	0.22
FGF17	0.24	0.19	IL29	0.26	0.15
FGF18	0.46	0.24	ILK	3.68	3.21
FGF19	0.26	0.24	INHA	0.26	0.18
FGF2	0.27	0.27	INSL3	0.38	0.41
FGF20	0.31	0.2	INSL4	0.36	0.5
FGF21	0.29	0.19	ITGA1	0.23	0.25
FGF22	0.28	0.19	JUN	2.25	3.41
FGF23	0.28	0.19	K6HF	3.79	2.79
FGF3	0.28	0.18	CD82	0.81	0.98
FGF4	0.28	0.22	KLK1	1.93	1.64
FGF5	0.28	0.22	KLK10	0.68	1.13
FGF6	0.28	0.22	KLK11	0.02	0.22
FGF7	0.28	0.09	KLK12	0.22	0.18
FGF8	0.28	0.21	KLK13	0.32	0.2
FGF9	0.28	0.18	**KLK14**	1.25	0.22
FHIT	0.29	0.18	KLK15	0.31	0.17
ARMC9	1.01	1.01	KLK2	1.62	2.15
FLJ25530	0.3	0.22	KLK3	0.28	0.18
FOLH1	0.27	0.08	KLK4	0.33	0.28
PAGE1	0.28	0.17	KLK5	0.37	0.52
PAGE4	0.28	0.2	KLK6	0.33	0.32
GGT1	0.31	0.19	KLK7	0.5	0.74
GNRH1	0.3	0.21	KLK8	0.09	0.39
GRP	0.31	0.19	KLK9	0.28	0.24
GSTP1	2.13	3.57	KRT1	0.27	0.24
HIF1A	0.32	0.28	KRT2A	0.23	0.15
HIP1	0.26	0.25	MAP2K4	2.06	2.2
HK2	0.29	0.25	MAP3K1	0.31	0.19
HK3	0.3	0.25	MAPK1	0.73	0.48
HRAS	0.29	0.21	MAPK10	0.23	0.19
KRT2B	0.32	0.12	MAPK11	0.31	0.22
IGF1	0.22	0.09	MAPK12	0.33	0.22
IGF1R	0.37	0.31	MAPK13	0.48	0.69
IGF2	0.37	0.75	MAPK14	0.33	0.29
IGFBP3	4.59	4.57	MAPK3	3.34	2.46
IGFBP6	1.56	1.97	MAPK4	0.31	0.23

APPENDIX 3 (Continued):

Oligo GEArray® Human Prostate Cancer Biomarkers Microarray Gene Expression - Set 2

GENES	CONTROL	Lutein 10μM	GENES	CONTROL	Lutein 10μM
MAPK6	2.02	0.91	REG3A	0.26	0.21
MAPK7	0.25	0.2	PART1	0.28	0.18
MAPK8	0.61	0.45	PATE	0.28	0.23
MAPK9	1.67	2.9	PAWR	1.45	0.78
MIB1	0.3	0.23	**PCA3**	0.22	0.26
MMP2	4.15	4.72	PCNA	4.3	4.09
MMP9	0.25	0.38	PGR	0.79	1.31
MSMB	0.27	0.22	PIAS1	0.27	0.26
MTSS1	0.25	0.22	PIAS2	0.33	0.3
MYC	3.49	3.8	PIK3CG	3.31	5.16
NCOA4	0.51	0.69	PLAU	5.34	5.22
NFKB1	2.39	2.63	PLG	0.37	0.46
NFKBIA	4.36	4.54	PPID	1.27	0.97
NKX3-1	0.29	0.23	TMEM37	1.63	1.59
NOX5	0.34	0.13	PRKCA	0.28	0.23
NR0B1	0.28	0.2	PRKCB1	0.31	0.19
NR0B2	0.28	0.1	PRKCD	1.88	2.41
NR1D1	0.25	0.35	PRKCE	0.25	0.22
NR1D2	0.29	0.25	PRKCG	0.47	0.76
NR1H2	0.26	0.23	PRKCH	0.71	0.99
NR1H3	0.42	0.88	PRKCI	0.47	0.36
NR1H4	0.28	0.24	PRKD3	3.09	3.61
NR1I2	0.37	0.41	PRKCQ	0.53	0.75
NR1I3	0.32	0.28	PRKCZ	0.33	0.35
NR2C1	0.35	0.29	PRKD1	0.32	0.22
NR2C2	0.3	0.27	PRKD2	0.27	0.2
NR2E1	0.28	0.19	PRL	0.28	0.22
NR2E3	0.49	0.52	PSAP	0.28	0.23
NR2F1	3.66	3.84	PSCA	1.52	0.98
NR2F2	0.23	0.28	PTEN	0.39	0.22
NR2F6	2.12	1.81	RARB	0.2	0.15
NR3C1	0.72	0.78	**RASSF1**	2.65	3.61
NR3C2	0.29	0.21	RB1	4.54	5.1
NR4A1	0.29	0.22	RNASEL	0.45	0.47
NR4A2	0.3	0.23	RNF14	0.35	0.5
NR4A3	0.3	0.24	ROBO2	0.36	0.18
NR5A1	0.25	0.22	SERPINA3	0.35	0.23
NR5A2	0.19	0.22	SHBG	0.28	0.22
NR6A1	0.26	0.19	SLC2A2	0.28	0.22
NTN4	0.45	0.69	SLC33A1	0.28	0.22
ODZ1	0.56	0.46	SLC43A1	0.28	0.21
PALM2-AKAP2	0.32	0.3	SOX2	0.28	0.2

APPENDIX 3 (Continued):

Oligo GEArray® Human Prostate Cancer Biomarkers Microarray Gene Expression - Set 2

GENES	CONTROL	Lutein 10μM
SRC	0.28	0.18
SRD5A2	0.28	0.16
HSPCB	5.46	5.26
STEAP1	2.89	3.82
STEAP2	1.57	0.69
TGFA	3.3	2.83
TGFB1	0.32	0.31
TGFB1I1	0.28	0.32
TGFB2	0.28	0.41
TGFB3	0.12	0.2
TIMP3	0.28	0.22
TNF	0.28	0.2
Pol1	0.28	0.18
PUC18	0.23	0.18
B2M	4.8	4.45
Blank	0	0
Blank	0	0
TNFSF10	0.36	0.28
TP53	0.32	0.25
TPM1	0.42	0.26
TPM2	0.71	0.8
18SrRNA	0.27	0.22
AS1R3	0.23	0.2
AS1R2	0.23	0.19
AS1R1	0.28	0.21
AS1	0.35	0.29
B2M	1.01	1
B2M	0.98	0.97
ACTB	5.51	5.11
TRPC6	0.41	0.33
TRPS1	0.3	0.34
TYK2	0.29	0.21
VEGFA	0.27	0.22
BAS2C	0.23	0.22
BAS2C	0.23	0.19
BAS2C	0	0.37
BAS2C	0.1	0
BAS2C	0	0

APPENDIX 4:

Oligo GEArray® Human Prostate Cancer Biomarkers Microarray Gene Expression - Set 3

GENES	CONTROL	Lutein 10μM	GENES	CONTROL	Lutein 10μM
RPS27A	0.79	0.82	CDK6	0.15	0.02
RPS27A	0.86	0.81	CDK7	0.37	0.4
AGR2	0.45	0.33	CDK8	0.23	0.07
AGTR2	0.25	0.48	CDK9	0.21	0.24
AIG1	0.42	0.46	CDKN1A	2.12	2.35
AKAP1	0.59	0.14	CDKN1B	0.11	0.36
AKT1	1.37	0.79	CDKN1C	1.56	0.84
APC	0.35	0.32	CDKN2A	0.63	0.55
APOC1	0.35	0.39	CDKN2B	0.58	0.42
GAPDH	1	1	CDKN2C	1.15	1.05
GAPDH	1	0.9	CDKN3	3.74	3.17
GAPDH	1	0.97	CHGA	0.14	0.19
RPS27A	0.98	0.91	CHGB	0.16	0.23
AR	0.95	0.78	CLDN3	0.34	0.5
BAK1	1.36	0.76	CLN3	2.4	2.63
BAX	0.52	0.41	CLU	3.29	4.51
BCL2	0.32	0.24	COL1A1	0.38	0.11
BCL2L1	0.44	0.43	COL6A1	3.11	3.99
BMP6	0.11	0.13	CYB5A	3.41	2.66
BRCA1	1.52	0.33	CYC1	4.83	3.19
CANT1	1.38	0.22	DAB2IP	0.42	0.5
CASP1	0.3	0.11	DAPK1	0.19	0.06
CASP3	0.39	0.12	DES	0.19	0.13
CASP7	0.11	0.25	DYNLL1	4.28	4.55
CAV1	3.31	2.41	E2F1	1.47	2.67
CCND1	4.41	4.33	EGF	0.35	0.17
CD164	0.1	0.42	**EGFR**	0.72	0.33
CD44	0.11	0.21	EGR3	0.27	0.12
CDH1	0.31	0.04	ELAC2	4.61	4.35
CDH10	0.3	0.06	ELL	0.15	0.21
CDH12	0.2	0.1	ENO1	5.29	5.28
CDH13	0.28	0.2	ENO2	0.58	0.83
CDH18	0.22	0.11	ENO3	0.44	0.32
CDH19	0.27	0.13	ERBB2	1.23	0.98
CDH20	0.25	0.24	MAPK15	0.42	0.01
CDH7	0.1	0.26	ESR1	0.95	0.64
CDH8	0.1	0.16	ESR2	0.31	0.16
CDH9	0.24	0.17	EZH1	0.47	0.31
CDK2	1.75	0.87	EZH2	0.59	0.25
CDK3	0.28	0.16	FASN	0.69	0.15
CDK4	4.23	4.14	FGF1	0.26	0.19
CDK5	0.64	0.2	FGF10	0.22	0.13

APPENDIX 4 (Continued):

Oligo GEArray® Human Prostate Cancer Biomarkers Microarray Gene Expression - Set 3

GENES	CONTROL	Lutein 10μM	GENES	CONTROL	Lutein 10μM
FGF11	0.19	0.08	IL12A	0.3	0.78
FGF12	0.3	0.05	IL1A	0.12	0.46
FGF13	0.24	0.29	IL1B	4.5	4.71
FGF14	0.26	0.25	IL2	0.09	0.12
FGF16	0.21	0.17	IL24	0.22	0.07
FGF17	0.41	0.28	IL29	0.2	0.06
FGF18	0.55	0.41	ILK	3.65	3.45
FGF19	0.32	0.39	INHA	0.16	0.29
FGF2	0.23	0.1	INSL3	0.42	0.4
FGF20	0.41	0.16	INSL4	0.4	0.43
FGF21	0.45	0.17	ITGA1	0.11	0.3
FGF22	0.33	0.14	JUN	2.52	3.34
FGF23	0.29	0.17	K6HF	3.69	2.79
FGF3	0.21	0.13	CD82	0.81	0.77
FGF4	0.22	0.19	KLK1	1.63	1.58
FGF5	0.26	0.18	KLK10	1.05	1.02
FGF6	0.18	0.19	KLK11	0.01	0.38
FGF7	0.15	0.02	KLK12	0.23	0.21
FGF8	0.36	0.2	KLK13	0.29	0.32
FGF9	0.34	0.25	**KLK14**	1.83	0.12
FHIT	0.16	0.13	KLK15	0.27	0.01
ARMC9	0.91	0.96	KLK2	1.68	2.28
FLJ25530	0.15	0.36	KLK3	0.26	0.11
FOLH1	0.2	0.28	KLK4	0.35	0.19
PAGE1	0.31	0.07	KLK5	0.2	0.42
PAGE4	0.22	0.11	KLK6	0.26	0.45
GGT1	0.17	0.1	KLK7	0.42	0.55
GNRH1	0.33	0.3	KLK8	0.16	0.38
GRP	0.44	0.05	KLK9	0.18	0.22
GSTP1	2.64	3.93	KRT1	0.23	0.3
HIF1A	0.23	0.13	KRT2A	0.2	0.28
HIP1	0.24	0.18	MAP2K4	2.22	2.41
HK2	0.21	0.3	MAP3K1	0.35	0.27
HK3	0.2	0.3	MAPK1	0.58	0.62
HRAS	0.39	0.47	MAPK10	0.21	0.13
KRT2B	0.26	0.2	MAPK11	0.25	0.23
IGF1	0.15	0.08	MAPK12	0.28	0.36
IGF1R	0.39	0.2	MAPK13	0.54	0.54
IGF2	0.24	0.7	MAPK14	0.35	0.18
IGFBP3	4.26	4.69	MAPK3	3.06	2.68
IGFBP6	1.07	1.88	MAPK4	0.3	0.14

APPENDIX 4 (Continued):

Oligo GEArray® Human Prostate Cancer Biomarkers Microarray Gene Expression - Set 3

GENES	CONTROL	Lutein 10μM	GENES	CONTROL	Lutein 10μM
MAPK6	2.33	0.7	REG3A	0.45	0.31
MAPK7	0.2	0.07	PART1	0.38	0.32
MAPK8	0.88	0.69	PATE	0.34	0.16
MAPK9	1.15	2.34	PAWR	1.67	0.61
MIB1	0.2	0.18	**PCA3**	0.34	0.08
MMP2	4.24	4.6	PCNA	4.13	4.39
MMP9	0.41	0.32	PGR	0.68	1.12
MSMB	0.26	0.09	PIAS1	0.23	0.37
MTSS1	0.34	0.14	PIAS2	0.36	0.2
MYC	3.8	3.51	PIK3CG	3.49	5.37
NCOA4	0.55	0.61	PLAU	5.19	5.25
NFKB1	2.5	2.86	PLG	0.43	0.57
NFKBIA	4.6	4.64	PPID	1.26	0.75
NKX3-1	0.38	0.15	TMEM37	1.56	1.65
NOX5	0.25	0.17	PRKCA	0.3	0.3
NR0B1	0.28	0.16	PRKCB1	0.32	0.1
NR0B2	0.27	0.06	PRKCD	1.67	2.11
NR1D1	0.3	0.29	PRKCE	0.24	0.08
NR1D2	0.26	0.19	PRKCG	0.74	0.88
NR1H2	0.46	0.27	PRKCH	0.97	0.79
NR1H3	0.3	0.67	PRKCI	0.21	0.12
NR1H4	0.3	0.19	PRKD3	3.15	3.72
NR1I2	0.32	0.32	PRKCQ	0.51	0.55
NR1I3	0.26	0.11	PRKCZ	0.34	0.37
NR2C1	0.32	0.27	PRKD1	0.3	0.2
NR2C2	0.31	0.25	PRKD2	0.25	0.31
NR2E1	0.2	0.12	PRL	0.31	0.34
NR2E3	0.64	0.47	PSAP	0.24	0.21
NR2F1	3.56	3.61	PSCA	1.33	0.61
NR2F2	0.26	0.12	PTEN	0.45	0.03
NR2F6	2.34	1.94	RARB	0.11	0.06
NR3C1	0.68	0.7	**RASSF1**	2.45	2.67
NR3C2	0.3	0.26	RB1	4.66	5.37
NR4A1	0.14	0.34	RNASEL	0.51	0.45
NR4A2	0.33	0.2	RNF14	0.32	0.36
NR4A3	0.4	0.2	ROBO2	0.29	0.13
NR5A1	0.32	0.41	SERPINA3	0.26	0.22
NR5A2	0.21	0.38	SHBG	0.18	0.2
NR6A1	0.2	0.24	SLC2A2	0.26	0.19
NTN4	0.5	0.51	SLC33A1	0.29	0.16
ODZ1	0.49	0.3	SLC43A1	0.18	0.2
PALM2-AKAP2	0.54	0.5	SOX2	0.23	0.17

APPENDIX 4 (Continued):

Oligo GEArray® Human Prostate Cancer Biomarkers Microarray Gene Expression - Set 3

GENES	CONTROL	Lutein 10μM
SRC	0.13	0.14
SRD5A2	0.1	0.09
HSPCB	5.55	5.23
STEAP1	2.97	3.62
STEAP2	1.78	0.59
TGFA	3.27	2.7
TGFB1	0.36	0.46
TGFB1I1	0.31	0.27
TGFB2	0.19	0.29
TGFB3	0.06	0.2
TIMP3	0.16	0.27
TNF	0.27	0.19
Pol1	0.14	0.12
PUC18	0.17	0.1
B2M	4.89	4.28
Blank	0	0
Blank	0	0
TNFSF10	0.46	0.35
TP53	0.31	0.1
TPM1	0.26	0.1
TPM2	0.67	0.78
18SrRNA	0.26	0.37
AS1R3	0.16	0.25
AS1R2	0.2	0.16
AS1R1	0.23	0.17
AS1	0.12	0.24
B2M	1.03	1.03
B2M	0.95	0.9
ACTB	5.39	5.64
TRPC6	0.29	0.46
TRPS1	0.46	0.45
TYK2	0.32	0.25
VEGFA	0.38	0.2
BAS2C	0.2	0.28
BAS2C	0.35	0.32
BAS2C	0	0.47
BAS2C	0	0
BAS2C	0	0