Chemopreventive Potential of an Ethyl Acetate Fraction from Curcuma Longa is Associated with Upregulation of p57kip2 and Rad9 in the PC-3M Prostate Cancer Cell Line

KVK Rao¹*, T Samikkannu¹, KB Dakshayani¹, X Zhang², SS Sathaye³, MA Indap⁴, Madhavan PN Nair¹

Abstract

Background: Turmeric (Curcuma longa) has been shown to possess anti-inflammatory, antioxidant and antitumor properties. However, despite the progress in research with C. longa, there is still a big lacuna in the information on the active principles and their molecular targets. More particularly very little is known about the role of cell cycle genes p57kip2 and Rad9 during chemoprevention by turmeric and its derivatives especially in prostate cancer cells. Methods: Accordingly, in this study, we have examined the antitumor effect of several extracts of C. longa rhizomes by successive fractionation in clonogenic assays using highly metastatic PC-3M prostate cancer cell line. Results: A mixture of isopropyl alcohol: acetone: water: chloroform: and methanol extract of C. longa showed significant bioactivity. Further partition of this extract showed that bioactivity resides in the dichloromethane soluble fraction. Column chromatography of this fraction showed presence of biological activity only in ethyl acetate eluted fraction. HPLC, UV-Vis and Mass spectra studies showed presence three curcuminoids in this fraction besides few unidentified components. Conclusions: From these observations it was concluded that the ethyl acetate fraction showed not only inhibition of colony forming ability of PC-3M cells but also up-regulated cell cycle genes p57kip2 and Rad9 and further reduced the migration and invasive ability of prostate cancer cells.

Keywords: Curcuma longa - ethyl acetate fraction - up-regulation - p57kip2 and Rad9 genes - invasive ability

Introduction

Chemoprevention has been acknowledged as an important and practical strategy for the management of cancer. Many naturally occurring substances present in the human diet have been identified as potential chemopreventive agents (Wattenberg, 1997; Krishnan et al., 1998; Rock, 1998; Vecchia & Tavani, 1998; Mukhtar & Ahmad, 1999; Aggarwal, 2008). The rhizome of the plant turmeric/ Curcuma longa has been used for centuries in traditional medicine and is known to have cancer preventive or therapeutic capabilities (Anand et al., 2008). Accordingly, turmeric fits very well and is an ideal candidate for chemoprevention by edible phytochemicals, which is now recognized as a plausible and cost-effective approach to reduce cancer morbidity and mortality by inhibiting precancerous events before the occurrence of clinical disease as well as for treatment of clinical disease (Thangapazham et al., 2006). However, despite the progress, still there is a big lacuna in the information on the active principles and their molecular targets of turmeric.

Prostate cancer is the second leading cause of cancer death in American men, behind only lung cancer. The American Cancer Society estimates that 28,660 men in the United States will die of Prostate Cancer in 2008 and accounts for about 9% of cancer related deaths in men (American Cancer Society, 2008). The epidemiological studies have shown significant correlations between prostate cancer incidence and dietary habits and the potential of dietary substances to act as chemopreventive agents against prostate cancer is increasingly appreciated (Syed et al., 2007: 2008). In this regard long-term well-designed and optimized intervention trials are required to delineate the potential clinical usefulness of C. longa both in normal populations as well as in high-risk groups and warrant further studies in the treatment and prevention of human neoplasm.

The p57kip2, one of CDK inhibitors of the Cip/Kip family, shares sequence homology with p27kip1 and p21 CIP1/WAF1 in the NH₂ terminal domain, which is involved in the binding to cyclin-CDK complexes (Lee et al., 1995; Matsuoka et al., 1995). Human p57kip2 is located

¹Department of Immunology, College of Medicine, Florida International University, Miami, Florida ²Department of Chemistry, Boston University, Boston, MA, USA ³Department of Pharmaceutical Sciences and Technology, Institute of Chemical Technology, Mumbai, ⁴Chemotherapy Division (Retd.), ACTREC, Kharghar, Navi Mumbai, India  *For correspondence: krao@fiu.edu

DOI:http://dx.doi.org/10.7314/APJCP.2012.13.3.1031
Role of p57kip2 and Rad9 during C. Longa Chemoprevention in PC-3M Cells
on chromosome 11p15.5, a region implicated in sporadic cancers and Beckwith-Wiedemann syndrome, a familial cancer syndrome (Matsuoka et al., 1995). The p57kip2 has been implicated in the modulation of many cellular events, including cell cycle control, differentiation, apoptosis, tumorigenesis, and development. Because of its chromosomal location, biochemical activities, and imprinting status, p57kip2 has been considered a candidate tumor suppressor gene (Zhang et al., 1997). HRad9 is an evolutionarily conserved human gene important for promoting resistance to DNA damage and regulating cell cycle checkpoints (Lieberman et al., 1996). The encoded protein can induce apoptosis (Komatsu et al., 2000), and regulate genomic stability (Hopkins et al., 2004). It has 3' to 5' exonuclease activity (Bessho et al., 2000), can bind p53 consensus DNA-binding sequences and up-regulate transcription of p21, as well as other downstream genes (Aiping et al., 2008). It can bind and stimulate activity of several DNA repair proteins involved primarily in base excision repair (Lieberman, 2006). AR can bind human Rad9 and represses androgen-induced AR transcription activity in prostate cancer cells thus regulating prostate function (Wang et al., 2004; Hsu et al., 2005). However, very little is known about the role of p57kip2 and Rad9 during chemoprevention by turmeric and its derivatives especially in prostate cancer cell lines.

*Curcuma longa* (turmeric) is known to have several components besides the major chemical component curcumin (diferuloylmethane) which may contribute to the observed beneficial effects, either alone or in combination with curcumin, although the precise identity and the role of the active fractions are yet to be elucidated. Further, it has been reported that curcumin alone is less effective than the combined in suppressing NF-KB activation (Sandu et al., 2007) suggesting that the unidentified other constituents in *C. longa* are critical for the total biological activity. However, despite the fact that the combination of compounds present in turmeric has higher efficacy than individual components, there is also a possibility of some components present in turmeric may not contribute for chemopreventive activity. Accordingly, in the present study, in order to increase the efficacy, we have made an attempt to identify the active fraction free from components non-contributory to chemoprevention by successive fractionation using different solvents, testing biological activity, identifying the components and see the effect of this active fraction on cell cycle associated genes p57kip2 and Rad9 as well as on invasive ability of highly metastatic PC-3M prostate cell line. Our results indicate that the fraction separated using ethyl acetate possess the biological activity, up-regulated the cell cycle associated genes and reduced invasiveness.

### Materials and Methods

#### Cell Culture

The effect of *C. longa* fractions were studied on highly metastatic human prostate cancer cell line PC-3M and normal prostate epithelial cells (RWPE-1), both obtained from American Type Culture Collection (ATCC). The PC-3M cells were grown in 25 cm² flasks / 60x15 mm tissue culture dishes containing RPMI 1640 (GIBCO/Invitrogen) with 10% fetal bovine serum (GIBCO/Invitrogen) and antibiotics. The prostate epithelial cells were grown in 25 cm² flasks / 60x15 mm tissue culture dishes containing keratinocyte Serum Free Medium (K-SFM)(GIBCO/ Invitrogen) supplemented with bovine pituitary extract (BPE)(0.05 mg/ml) and epidermal growth factor (EGF) (5 ng/ml). The cells were maintained in a humidified, 95% air and 5% CO₂ atmosphere incubator at 37 °C.

### Extraction, partition and column chromatography of *C. longa*

The ground powder (100g) of dried rhizomes of *C. longa* was extracted using a combination of isopropyl alcohol: acetone: water: chloroform: and methanol in the ratio of 4:4:6:3:3 for 48 h at room temperature in dark. The extract was filtered and the residue left was re-extracted with the same solvents. The filtrates were combined, concentrated under reduced pressure at low temperature (40-45 °C) in rotary vacuum evaporator. The dried extract was stored at 40 °C for further analysis. A small quantity of this extract was dissolved in dimethylsulfoxide (DMSO) and tested for biological activity and another portion of this extract was partitioned with water: dichloromethane (1:1). The aqueous layer was concentrated under vacuum and dried. The organic (dichloromethane) layer was concentrated under reduced pressure at 60 °C in rotary vacuum evaporator. Also a third interface fraction was obtained between the aqueous and organic layer. All the three fractions were dissolved in DMSO and tested for biological activity. Only the dichloromethane fraction showed significant inhibitory effect on colony formation in clonogenic assays suggesting that potential chemopreventive activity resides in dichloromethane soluble fraction. This dichloromethane fraction was subjected to column chromatography (silica gel, 200-400 mesh, column size 20 cm) and eluted successively with hexane, ethyl acetate, methanol and ethanol. All the four elutes were evaporated to dryness and dissolved in DMSO and tested for biological activity. Results indicated that only the ethyl acetate fraction showed significant inhibitory effect on colony formation in clonogenic assays and accordingly utilized for identification of the components present and for mechanism studies.

### Analysis and identification of ethyl acetate fraction

A solution of the extract in dimethylsulfoxide (10 mg/ml) was diluted about 1:100 in methanol and water (v/v, 1:1). HPLC-DAD-MS analysis was performed with an Agilent 1100 liquid chromatography system consisting of an automatic injector, a gradient pump, a Hewlitt-Packard series 1100 diode array detector, and an Agilent series 1100 VL on-line atmospheric pressure ionization electrospray ionization mass spectrometer. Separations were done on a C18 reversed phase column (Vydac 218TP52, 2.1 mm diameter x 250 mm; 5 μm particle size). The column was eluted at a flow rate of 0.35 ml/min with a gradient of water with 1% (v/v) formic acid (A) and acetonitrile with 1% (v/v) formic acid (B) using the following elution program: 0 min, 95% A, 5% B, 0-40 min, a linear gradient
to 30% A, 70% B, 40-45 min, a linear gradient elution to 15% A, 85% B, 45-50 min, isocratic elution at 15% A, 85% B; 50-55 min gradient elution to 95% A, 5% B, and re-equilibration with the latter solvent for 15 min. The mass spectrometer was run in the positive ion mode.

Clonogenic Assays

Clonogenic assays using logarithmically growing cells were performed as described earlier (Panandiker et al., 1992; 1994; Mahudawala et al., 1999; Nair et al., 2004; Rao et al., 2004). In brief, approximately 1000 cells obtained from sub-confluent culture flasks were seeded per 60 mm tissue culture dishes in 5 ml of medium (five dishes per point). Twenty four hours after seeding the cells, test compounds were added at selected concentrations to the medium. Control cultures received only solvent in the place of test chemical. dimethylsulfoxide (DMSO) served as the vehicle to dissolve compounds at a final concentration of 0.4% in the culture medium, volume per volume. Preliminary experiments have shown that 0.4% volume per volume DMSO has no effect on cell survival. Dishes were returned to the incubator for up to 7 to 8 days and surviving cells were allowed to form colonies. When the colonies were discrete and well defined, the dishes were washed with PBS solution, fixed with methanol, stained with Giemsa and allowed to dry. The colonies per dish were counted using Computer based Quantity One BIO-RAD version 4.6.3.Windows and Macintosh Software. The assays were repeated two to three times and depicted for one assay. The results were the same in other assays.

RNA extraction and quantitative real-time PCR (QRT-PCR)

The total RNA from approximately 1 x 10^6 PC-3M cells both control and curcumin treated was extracted using RNA purification Qiagen kit (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer’s instructions. The RNA was eluted, quantified by absorbance at 260 nm and stored at -80 °C. Total RNA (5 μg) from all the samples were reverse transcribed using the high capacity cDNA reverse transcription kit (Applied Biosystems, Foster city, CA). The cDNA was stored at -20 °C until further analysis. Expression of mRNAs for both Rad9 (Hs00270240_m1) and p57 kip2 (Hs00175938_m1) were measured by one-step real-time reverse transcription-polymerase chain reaction (RT-PCR) using TaqMan Gene Expression Assays (Applied Biosystems) (For pre-designed assays, the manufacturer does not provide the primer and probe sequences). The probe anneals to a complementary sequence between the forward and reverse primer sites on the target. When the DNA polymerase extends the upstream primers and encounters the downstream probe, the 51 to 31 nucleotide activity of the polymerase cleaves the probe and the reporter fluorophore is released into the solution and the amount of fluorescence is measured at each amplification cycle (Cunningham, 2001) using Mx3000P Instrument. The PCR cycle at which fluorescence measured by the instrument reaches instrument defined threshold value (Ct or threshold cycle) was measured to quantify the target gene. β -actin was used as an internal control and the primers used were: Forward (5’-TGACGGGGTCACCCACACTGTGCGCCATCTA-3’) Reverse (3’-AGTCATAGTCCGCTTAAGACGATTGT CGGT-3’). All the results were expressed as the ratio of the expression of the target gene normalized to β -actin gene in treated cells compared to the normalized expression of the target genes in untreated cells (Shively et al., 2003).

Cell Invasion Assay

The invasive activity of PC-3M prostate cancer cells and normal prostate epithelial cells in vitro was tested using a QCMTM 24-Well Collagen-Based Cell Invasion Assay kit (Chemicon International, Inc.,). The invasion assay was performed using a 24-well tissue culture plate with 12 cell culture inserts. The insert contains an 8 μm pore size polycarbonate membrane coated with a thin layer of polymerized collagen. Prior to initiating assay, the plates and reagents were brought to room temperature. The collagen layer was rehydrated for 15-30 minutes at room temperature by adding 300 μl of pre-warmed serum free media to the interior of the inserts. After rehydration, 250 μl of media was carefully removed from the inserts without disturbing the collagen-coated membrane. A cell suspension containing 1.0 x 10^6 cells/ml from serum starved media was prepared in chemo-attractant-free media and 250 μl of prepared cell suspension was added to each insert. To the lower chamber 500 μl of media in the presence of chemo-attractant was added. Ethyl acetate extract at 5 μg/ml was added to the experimental inserts. Controls received only solvent in the place of test chemical. Dimethylsulfoxide (DMSO) served as the vehicle to dissolve the compounds at a final concentration of 0.4% in the medium, volume per volume. The plates were covered and incubated for 72 hours at 37 °C in a CO2 incubator (5% CO2). After the incubation period, the media was discarded and the cells were stained and inserts were dippled into a beaker of water several times to rinse. While the inserts were still moist, the non-invading cells and collagen layer were gently removed from the interior of the inserts using a cotton-tipped swab, air dried and cells that migrated through the porous membrane and adhered to the lower surface were counted. The experiments were done in quadruplicate wells.

Statistics

The results were expressed as mean ± SD and significance was evaluated using Student’s t-test (GraphPad Software). P values of ≤ 0.05 and less were considered significant. The final values were expressed as either per cent relative plating efficiency or per cent decrease in colony formation compared to solvent controls.

Results

Extraction, partition, column chromatography and identification of C. longa derivatives

Chemoprevention has been acknowledged as an important and practical strategy for the management of...
cancer. Recent research provides evidence that many daily consumed dietary compounds possess cancer preventive properties. From this point of view, we have selected 13 plant materials, which are in common use either as dietary supplements or traditional medicine and extracted with a mixture of isopropyl alcohol, acetone, water, chloroform and methanol for testing the growth inhibitory effects on prostate cancer cell lines (unpublished observations). Out of 13 lyophilized plant extracts evaluated for growth inhibitory effects, *Curcuma longa* /turmeric powder extract showed significant inhibitory effect on the colony-forming ability of the highly metastatic PC-3M prostate cancer cell line at 20 and 40 $\mu$g/ml concentrations. In order to see whether whole turmeric extract is effective or only a fraction, the turmeric extract was partitioned with dichloromethane: water (1:1) as described in the Materials and Methods section. The three fractions obtained were tested for biological activity using clonogenic assays. Only the dichloromethane fraction showed major inhibitory effect on colony formation in clonogenic assays suggesting that potential chemopreventive activity resides in dichloromethane soluble fraction. The water and Interface fractions showed relatively marginal effects. To increase the efficacy, the dichloromethane fraction was further subjected to column chromatography (silica gel, 200-400 mesh column size 20 cm) and eluted successively with hexane, ethyl acetate, methanol and ethanol. All the four elutes were evaporated to dryness and dissolved in DMSO and tested for biological activity. Results showed that only the ethyl acetate eluted fraction showed significant inhibitory effect on colony formation in clonogenic assays (Table 1 and Figure 1). Accordingly this fraction was utilized for identification of the components present as well as for mechanism studies. The HPLC profile of the ethyl acetate fraction showed that the main components present are curcuminoids (curcumin, etc.).

**Table 1. Effect of Hexane, Ethyl Acetate, Methanol, and Ethanol Eluted Fractions from Dichloromethane Extract of *C. longa* on Highly Metastatic PC-3M Cell Line in Clonogenic Assays.**

| Treatment | Hexane | Ethyl Acetate | Methanol | Ethanol | (µg/ml) |
|-----------|--------|---------------|----------|---------|---------|
| DMSO      | 354±13.8 | 432±23.6      | 432±23.6 | 432±23.6 | 0.625   |
| 0.625     | 393±4.8  | 397±15.1      | 397±15.1 | 397±15.1 | 1.25    |
| 2.5       | 391±27.6 | 371±42.5      | 492±25.5 | 531±20.2 | 5       |
| 5         | 360±21.8 | 164±22.7      | 472±35.9 | 521±20.5 | 10      |

* *APE-Absolute Plating Efficiency. $p < 0.022$; $p < 0.0004$; $p < 0.0001$*

**Figure 1. Representative Petri Dishes Showing the Effect of the Purified Fractions from *C. longa* on Highly Metastatic PC-3M Prostate Cancer Cell Line in Clonogenic Assays.**

A. DMSO control; B. Hexane fraction; C. Ethyl acetate fraction; D. Methanol fraction; E. Ethanol fraction.

**Figure 2. Effect of Ethyl Acetate Fraction from *C. longa* on Highly Metastatic PC-3M Cell Line and Normal Prostate Epithelial Cells in Clonogenic Assays.**

**Figure 3. Effect of Ethyl Acetate Fraction from *C. longa* on p57kip2 and Rad9 Expression in Highly Metastatic PC-3M cell line.**

* Not significant; **$p<0.02$; ***$p<0.009$

**Figure 4. Effect of Ethyl Acetate Fraction from *C. longa* on the Invasive Ability of Highly Metastatic PC-3M Cell Line and Normal Prostate Epithelial Cells (x100).**

A. PC-3M cells; B. Normal prostate epithelial cells; C. % Relative number of migrated cells in PC-3M cell line. *p<0.0002

successively with hexane, ethyl acetate, methanol and ethanol. All the four elutes were evaporated to dryness and dissolved in DMSO and tested for biological activity. Results showed that only the ethyl acetate eluted fraction showed significant inhibitory effect on colony formation in clonogenic assays (Table 1 and Figure 1). Accordingly this fraction was utilized for identification of the components present as well as for mechanism studies. The HPLC profile of the ethyl acetate fraction showed that the main components present are curcuminoids (curcumin,
demethoxycurcumin, and bisdemethoxycurcumin) (Rao et al., 2011). The UV-Vis and mass spectra of peaks 1 and 2 further confirmed that peak 1 consists of a mixture of curcumin and demethoxycurcumin and that peak 2 contains bisdemethoxycurcumin (Rao et al., 2011). Besides main components of all curcuminoids, presence of some minor components was also observed (Rao et al., 2011) in the ethyl acetate fraction. Further studies are required to identify these components and their biological significance.

**Effect of Ethyl acetate Fraction on PC-3M and Normal Prostate Epithelial Cells**

The ethyl acetate fraction showed significant inhibitory effects on the colony-forming ability of the highly metastatic PC-3M prostate cancer cell line at different concentrations tested (Figure 2). On the normal prostate epithelial cells ethyl acetate fraction showed similar effects but magnitude was less.

**Up-regulation of p57kip2 and Rad9 genes**

Data presented in Figures 3 show the dose dependent effect of ethyl acetate fraction on p57kip2 and Rad9 gene expression in PC-3M cell line. PC-3M cells were treated with ethyl acetate fraction at different concentrations for 24 h. RNA was extracted and reverse transcribed and both p57kip2 and Rad9 gene expression was quantified by real time PCR using β-actin as a house keeping gene. PC-3M cells treated with ethyl acetate fraction both at 4 and 6μg/ml for 24 h showed significant up-regulation of about two fold higher the amount of both p57kip2 and Rad9 genes when compared to corresponding controls. No significant difference in gene expression was observed at 2 μg/ml concentrations for both the genes (Figure 3).

**Up-regulation of p57kip2 and Rad9 is associated with decreased the invasive ability**

To understand functional significance of up-regulation of both p57kip2 and Rad9 during chemoprevention by ethyl acetate fraction, Collagen-Based Cell Invasion Assay was performed. The ability of cells to invade collagen and migrate to the underside of the inserts was determined by a 72-hour response to medium containing chemottractant i.e. 10% FBS in the lower chamber. In the presence of ethyl acetate fraction, significantly fewer cells migrated to the underside compared with the absence of ethyl acetate fraction (Figure 4) suggesting that both p57kip2 and Rad9 expression is associated with the reduced migration and invasive ability of prostate cancer cells. No invasive ability was observed either in the presence or absence of ethyl acetate fraction in normal prostate epithelial cells.

**Discussion**

*Curcuma longa* or turmeric, a widely cultivated tropical plant has been used since ancient times as a spice, as a beauty care agent and therapeutically for a wide range of ailments and as well as in traditional medicine (Kunnunakkara et al., 2008). Turmeric is traditionally known as a blood purifier and is reported to be useful for the common cold, intermittent fevers, affictions of the liver, indolent ulcer and wound healing. It has been found to possess anti-inflammatory and antioxidant activities and chemopreventive activity for a wide variety of cancers like colon, breast, prostate, esophagus, lung, oral and has potential as an antiviral and antibacterial agent (Mazumder et al., 1995; Anto et al., 1996; Aggarwal et al., 2003; Doria & Aggarwal, 2004; Duvoix et al., 2005; Olszanecki et al., 2005; Park et al., 2005; Alpers, 2008). Recently Aggarwal and co-workers using a commercially available curcumin mix reported that different analogs of curcumin present in turmeric showed variable anti-inflammatory and anti-proliferative activities (Sandur et al., 2007). Curcumin or diferuloylmethane, a major component present in turmeric is a powerful antioxidant and is linked with the suppression of mutagenesis, inhibited nuclear factor-κB (NF-κB) activation, suppressed cyclin D1 and anti-apoptotic gene products, induced cytochrome C release, activated caspases and have anti-angiogenic effects through down-regulation of vascular endothelial growth factor (VEGF) (Choudhuri et al., 2005; Aggarwal et al., 2006; Aggarwal et al., 2007; Liu et al., 2007; Shankar & Srivastava, 2007). Curcumin is currently in clinical trials for treatment of various cancers (Sharma et al., 2004; Garcea et al., 2005; Dhillon et al., 2006; Rafailov et al., 2007) and for Alzheimer’s disease (Yang et al., 2005).

In the present study *Curcuma longa* turmeric powder extract showed significant inhibitory effect on the colony-forming ability of the highly metastatic PC-3M prostate cancer cell line. Further, partition and fractionation studies showed that only the ethyl acetate eluted fraction possessed significant inhibitory effect on colony formation in clonogenic assays (Table 1 and Figure 1). Accordingly this fraction was utilized for identification of the components present as well as for mechanism studies. HPLC, UV-Vis and mass spectra analysis showed the presence of three main curcuminoids namely curcumin, demethoxycurcumin, and bisdemethoxycurcumin in ethyl acetate fraction (Rao et al., 2011). Besides main components of all curcuminoids, the presence of some minor components was also observed (Rao et al., 2011) in the ethyl acetate fraction (Rao et al., 2011). Besides main components of all curcuminoids, the presence of some minor components was also observed (Rao et al., 2011) in the ethyl acetate fraction (Rao et al., 2011). Besides main components of all curcuminoids, the presence of some minor components was also observed (Rao et al., 2011) in the ethyl acetate fraction (Figure 4) suggesting that both p57kip2 and Rad9 genes when compared to corresponding controls. No significant difference in gene expression was observed at 2 μg/ml concentrations for both the genes (Figure 3).

Over-expression of p57kip2 has been shown to cause cell growth arrest and senescent phenotype in many cell types (Jin et al., 2004; Williams et al., 2005). P57kip2 has been considered a candidate tumor suppressor gene due to its location in the genome, biochemical activities, and imprinting status (Jin et al., 2008). Previously, many studies have focused on p21cip1/Waf1 or p27kip1 whereas little is known about the biological function of p57kip2 during tumorigenesis and cancer progression (Jin et al., 2008). Recently, it has been shown that the expression of p57kip2
is significantly decreased in human prostate cancer (Jin et al., 2008) and the over-expression of p57kip2 in prostate cancer cells significantly suppressed cell proliferation and reduced invasive ability (Jin et al., 2008). Further, in LNCaP cells, over-expression of p57kip2 inhibited tumor formation in nude mice and the prostate of p57kip2 knockout mice developed prostatic intraepithelial neoplasia and adenocarcinoma suggesting that p57kip2 is an important gene in prostate cancer tumorigenesis, and the p57kip2 pathway may be a potential target for prostate cancer prevention and therapy, more specifically, during turmeric chemoprevention (Jin et al., 2004; 2008; Williams et al., 2005). The Rad9 gene has many functions that could bear on carcinogenesis, including a role in maintaining genome integrity and regulating cell cycle checkpoints (Lieberman et al., 1996; Bessho & Sancar, 2000; Komatsu et al., 2000; Hopkins et al., 2004; Aiping et al., 2008). Rad9 protein can bind AR and this protein-protein interaction represses the ability of testosterone to induce a conformational change in the receptor, to activate a receptor transcription regulatory function, and subsequently to express downstream target genes critical for proper prostate function (Wang et al., 2004; Hsu et al., 2005; Lieberman, 2006). In our studies, PC-3M cells treated with ethyl acetate fraction both at 4 and 6µg/ml for 24 h showed significant up-regulation of about two fold higher the amount of both p57kip2 and Rad9 genes when compared to corresponding controls (Figures 3). No significant difference in gene expression was observed at 2 µg/ml concentration for both the genes (Figures 3). Therefore, these results suggest that up-regulation of both p57kip2 and Rad9 genes are associated with inhibition of cell proliferation and accordingly reduced colony formation in cells treated with ethyl acetate fraction. P57kip2 has important functions, such as binding to proliferating cell nuclear antigen to prevent DNA replication and inhibit cell transformation (Tsugui et al., 2000). It is also involved in glucocorticoid-induced anti-proliferation (Samuelsson et al., 1999). Recently, it has been shown that over-expression of p57kip2 in prostate cancer cells significantly suppressed the cell proliferation and arrested the cell cycle at GO-G1 stage (Jin et al., 2008). Studies are in progress to see whether similar mechanisms are operative during PC-3M cell exposure to ethyl acetate fraction.

Invasion through the extracellular matrix is an important step in tumor metastasis. By releasing proteolytic enzymes such as MMPs and collagenases, cancer cells are able to breach the membrane and penetrate the blood vessel wall and invade (Albini, 1998). Collagen, the primary structural element of the basement membrane and tissue scaffolding protein, represents the main deterrent in the migration of tumor cells. The ability to study cell invasion through a collagen barrier, is of vital importance for developing possible metastatic inhibitors and therapeutics. The objective of our study is to see whether up-regulation of both p57kip2 and Rad9 genes in PC-3M cells treated with ethyl acetate is associated with a decrease in the invasive potential of the tumor cells. Consistent with the known metastatic potential, the untreated PC-3M cells were relatively more invasive compared to the cells treated with ethyl acetate fraction.

In the presence of ethyl acetate fraction, very few cells migrated to the underside of the insert compared to the cells in the absence of ethyl acetate fraction (Figure 4). These results indicate that inhibition of colony forming ability of PC-3M cells by ethyl acetate fraction is not only associated with up-regulation of p57kip2 and Rad9 but also reduced the migration and invasive ability of prostate cancer cells. Normal prostate epithelial cells were not invasive at all either in the presence or absence of ethyl acetate fraction (Figure 4). It is known that invasiveness of cells through a gel of basement membrane proteins correlates well with metastatic potential in vivo (Dedhar et al., 1993; Albini, 1998).

Acknowledgments

This study was supported by grants from the National Institutes Health (NIH): 1R01MH085259, 1R37DA025576, 5RO1DA021537, and 1RO1DA027049 to Prof. Madhavan Nair. The authors would like to thank Prof. Richard Laursen, Department of Chemistry, Boston University, Boston, USA for his contributions to the analysis of C. longa extract and Dr. Steven J. Melnick, Miami Children’s Hospital for photomicrographs. The authors declare that they have no conflicts of interest. Requests for reprints to Madhavan P.N. Nair, Ph.D. Professor and Chair of Immunology, Associate Dean of Bio-Medical Research, College of Medicine, University Park, 11200 S.W.8th Street, Miami, FL-33199, Tel: 305-348-0570, Email: nairm@fiu.edu.

References

Aggarwal BB (2008). The past, present and future of multi-targeted cancer treatment “Naturally”: Food for thought. Cancer Lett, 269, 187-8.

Aggarwal BB, Bhatt ID, Ichikawa H, et al (2006). Curcumin-Biological and Medicinal Properties, The CRC Press, Boca Raton, FL, ?, 297-368.

Aggarwal BB, Kumar A, Bharti AC (2003). Anticancer potential of curcumin: preclinical and clinical studies. Anticancer Res, 23, 363-98.

Aggarwal BB, Surh YH, Shishodia S (2007). The molecular targets and therapeutics of curcumin in health and disease. Advances in Experimental Medicine and Biology, vol. 995, Springer Publication.

Aiping Z, Charles XZ, Lieberman HB (2008). Rad9 Has a Functional Role in Human Prostate Carcinogenesis. Cancer Res, 68, 1267-74.

Albini A (1998). Tumor and endothelial cell invasion of basement membranes. The Matrigel chemoinvasion assay as a tool for dissecting molecular mechanisms. Pathol Oncol Res, 4, 230-2.

Alpers DH (2008). The potential use of curcumin in management of chronic disease: too good to be true ?. Curr Opin Gastroenterol, 24, 173-5.

American Cancer Society: What are the key statistics about prostate cancer, Cancer Reference Information 2008.

Anand P, Sundaram C, Jhurani S, Kunnumakkara AB, Aggarwal BB (2008). Curcumin and cancer: An “old-age” disease with an “age-old” solution. Cancer Lett, 267, 133-64.

Anto RJ, George J, Babu KV, Rajasekharan KN, Kuttan R (1996).
Role of p57kip2 and Rad9 during C. Longa Chemoprevention in PC-3M Cells

DOI:http://dx.doi.org/10.7314/APJCP.2012.13.3.1031

Antimutagenic and anticarcinogenic activity of natural and synthetic curcuminooids. *Mutat Res*, 370, 127-31.

Bessho T, Sancar A (2000). Human DNA damage checkpoint protein hRAD9 is a 35 to 55 exonuclease. *J Biol Chem*, 275, 7451-4.

Choudhuri PST, Das T, Sa G (2005). Curcumin selectively induces apoptosis in deregulated cyclin D1-expressed cells at G2 phase of cell cycle in a p53-dependent manner. *J Biol Chem*, 20, 20059-68.

Cunningham B (2001). Assessing differential gene expression. *The Scientist*, 15, 27-31.

Dedhar S, Saulnier R, Nagle R, Overall CM (1993). Specific alterations in the expression of alpha3beta1 and alpha6beta4 integrins in highly invasive and metastatic variants of human prostate carcinoma cells selected by in vitro invasion through reconstituted basement membrane. *Clin Exp Metastasis*, 11, 391-400.

Dhillon N, Aggarwal BB, Li L, et al (2006). Phase II trial of curcumin (diferuloylmethane), an NF-κB inhibitor, in patients with advanced pancreatic cancer. *J Clin Oncol*, 24, 14151-6.

Dorai T, Aggarwal BB (2004). Role of chemopreventive agents in cancer therapy. *Cancer Lett*, 215, 129-40.

Duvoix A, Blasius R, Delhalle S, et al (2005). Chemopreventive and therapeutic effects of curcumin. *Cancer Lett*, 223, 181-90.

Garcea G, Berry DP, Jones DJ, et al (2005). Consumption of the chemopreventive agent curcumin by cancer patients: assessment of curcumin levels in the colorectum and their pharmacodynamic consequences. *Cancer Epidemiol Biomarkers Prev*, 14, 120-5.

Hopkins KM, Au erbach W, Wang XY, et al (2004). Deletion of mouse Rad9 causes abnormal cellular responses to DNA damage, genomic instability, and embryonic lethality. *Mol Cell Biol*, 16, 7235-48.

Hsu CL, Chen YL, Ting HJ (2005). Androgen receptor (AR) NH2- and COOH-terminal interactions result in the differential influences on the AR-mediated transactivation and cell growth. *Mol Endocrinol*, 19, 350-61.

Jin RJ, Lho Y, Wang Y, et al (2008). Down-regulation of p57kip2 Induces Prostate Cancer in the Mouse. *Cancer Res*, 68, 3601-8.

Jin RJ, Wang Y, Masumori N, et al (2004). NE-10 neuroendocrine cancer promotes the LNCaP xenograft growth in castrated mice. *Cancer Res*, 64, 5489-95.

Komatsu K, Miyashita T, Hang H, et al (2000). Human homologue of S. pombe Rad9 interacts with Bcl-2/Bcl-XL and promotes apoptosis. *Nat Cell Biol*, 2, 1-6.

Krishnan K, Ruffin MT, Brenner DE (1998). Cancer chemoprevention: A new way to treat cancer before it happens. *Primary Care*, 25, 361-79.

Kunnumakkara AB, Anand P, Aggarwal BB (2008). Curcumin inhibits proliferation, invasion, angiogenesis and metastasis of different cancers through interaction with multiple cell signaling proteins. *Cancer Lett*, 269, 199-225.

Lee MH, Reynisdottir I, Massague J (1995). Cloning of p57kip2, a cyclin-dependent kinase inhibitor with unique domain structure and tissue distribution. *Genes Dev*, 9, 639-49.

Lieberman HB, Hopkins KM, Nass M, Demetrick D, Davey S (1996). A human homolog of the Schizosaccharomyces pombe rad9+ checkpoint control gene. *Proc Natl Acad Sci USA*, 93, 13890-5.

Lieberman HB (2006). Rad9, an evolutionarily conserved gene with multiple functions for preserving genomic integrity. *J Cell Biochem*, 97, 690-7.

Liu E, Wu J, Cao W, et al (2007). Curcumin induces G2/M cell cycle arrest in a p53-dependent manner and upregulates ING4 expression in human glioma. *J Neurooncol*, 85, 263-70.

Mahudawala DM, Redkar AA, Wagh A, Gladstone B, Rao KV (1999). Malignant transformation of Syrian hamster embryo (SHE) cells in culture by malachite green: An agent of environmental importance. *Indian J Exp Biol*, 37, 904-18.

Matsuoka S, Edwards MC, Bai C, et al (1995). p57kip2, a structurally distinct member of the p21Cdk inhibitor family, is a candidate tumor suppressor gene. *Genes Dev*, 9, 650-62.

Mazumder A, Raghavan K, Weinstein J, Kohn KW, Pommier Y (1995). Inhibition of human immunodeficiency virus type-1 integrase by curcumin. *Biochem Pharmacol*, 49, 1165-70.

Mukhtar H, Ahmad N (1999). Cancer chemoprevention: future holds in multiple agents. *Toxicol Appl Pharmacol*, 158, 207-10.

Nair HK, Rao KV, Aalinkel R, et al (2004). Inhibition of prostate cancer cell colony formation by the flavonoid quercetin correlates with modulation of specific regulatory genes. *Clin Diag Lab Immunol USA*, 11, 63-9.

Olszanecki R, Jawien J, Gadja M, et al (2005). Effect of curcumin on atherosclerosis in apoE/IDL-R-double knockout mice. *J Physiol Pharmacol*, 56, 627-35.

Panandiker A, Fernandes C, Rao KV (1992). The cytotoxic properties of malachite green are associated with the increased demethylase, aryl hydrocarbon hydroxylase and lipid peroxidation in primary cultures of Syrian hamster embryo cells. *Cancer Lett*, 67, 93-101.

Panandiker A, Maru GB, Rao KV (1994). Dose-response effects of malachite green on free radical formation, lipid peroxidation and DNA damage in Syrian hamster embryo cells and their modulation by antioxidants. *Carcinogenesis*, 15, 2445-8.

Park BS, Kim JG, Kim MR, et al (2005). Constituents inhibit sortase A and Staphylococcus aureus cell adhesion to fibronectin. *J Agric Food Chem*, 53, 9005-9.

Rafailov S, Cammack S, Stone BA, Katz AE (2007). The role of zymflamend, an herbal anti-inflammatory, as a potential chemopreventive agent against prostate cancer: a case report. *Integr Cancer Ther*, 6, 74-6.

Rao KV, Schwartz SA, Nair HK, et al (2004). Plant derived products as a source of cellular growth inhibitory phytochemicals on PC-3M, DU-145 and LNCaP prostate cancer cell lines. *Curr Sci*, 87, 1585-8.

Rao KV, BouklI NM, Samikkannu T, et al (2011). Proteomic profiling and cytotoxic effect of Curcuma longa on prostate cancer. *The Open Proteomics J*, 14, 1-11.

Rao CL (1998). Nutritional factors in cancer chemoprevention. *The Scientist*, 97, 27-31.

Sandur SK, Pandey MK, Sung B, et al (2007). Curcumin, demethoxycurcumin, bisdemethoxycurcumin, tetrahydrocurcumin, and turmerones differentially regulate anti-inflammatorny and antiproliferative responses through a ROS-independent mechanism. *Carcinogenesis*, 8, 1765-73.

Samuelsson MKR, Pazirandeh A, Davani B, Okret S (1999). p57kip2, a glucocorticoid-induced inhibitor of cell cycle progression in HeLa cells. *Mol Endocrinol*, 13, 1811-22.

Sharma RA, Euden SA, Platton SL, et al (2004). Phase I clinical trial of oral curcumin: biomarkers of systemic activity and compliance. *Clin Cancer Res*, 10, 6847-54.

Shively CA, Mirkes SJ, Lu NZ, Henderson JA, Bethca CL (2003). Soy and social stress affect serotonin neurotransmission in primates. *Pharmacogenomics*, 3, 114-21.

Syed DN, Khan N, Afaq F, Mukhtar H (2007). Chemoprevention of prostate cancer through dietary agents: progress and promise. *Cancer Epidemiol Biomarkers Prev*, 16, 2193-204.

Syed DN, Suh Y, Afaq F, Mukhtar H (2008). Dietary agents
KVK Rao et al

for chemoprevention of prostate cancer. Cancer Lett, 265, 167-76.

Thangapazham RL, Sharma A, Maheshwari RK (2006). Multiple molecular targets in cancer chemoprevention by curcumin. AAPSJ, 8, 443-9.

Tsugu A, Sakai K, Dirks PB, et al (2000). Expression of p57(KIP2) potently blocks the growth of human astrocytomas and induces cell senescence. Am J Pathol, 57, 919-32.

Vecchia CL, Tavani A (1998). Fruits and vegetables, and human cancer. Eur J Cancer, 7, 3-8.

Wang L, Hsu CL, Ni J (2004). Human checkpoint protein hRad9 functions as a negative coregulator to repress androgen receptor transactivation in prostate cancer cells. Mol Cell Biol, 24, 2202-13.

Wattenberg LW (1997). An overview of chemoprevention: current status and future prospects. Proc Soc Exp Biol Med, 216, 133-41.

Williams K, Fernandez S, Stien X, et al (2005). Unopposed c-MYC expression in benign prostatic epithelium causes a cancer phenotype. Prostate, 63, 369-84.

Yang F, Lim GP, Begum AN, et al (2005). Curcumin inhibits formation of amyloid beta oligomers and fibrils, binds plaques, and reduces amyloid in vivo. J Biol Chem, 280, 5892-901.

Zhang P, Liegeois NJ, Wong C, et al (1997). Altered cell differentiation and proliferation in mice lacking p57KIP2 indicates a role in Beckwith-Wiedemann syndrome. Nature, 387, 151-8.