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Review Article

Pomegranate fruit as a lung cancer chemopreventive agent

Naghma Khan, Hasan Mukhtar*
*Department of Dermatology, University of Wisconsin, Madison, WI 53706, USA. *Correspondence: e-mail: hmukhtar@wisc.edu*

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Abstract

Lung cancer is one of the major causes of cancer-related deaths, and there is therefore a need to develop new approaches to control this leading cancer threat. Studies from our lab have shown that pomegranate fruit extract (PFE) possesses antioxidant, anti-inflammatory and antitumor properties. We have shown that treatment of A549 human lung carcinoma cells with PFE (50-150 μg/ml) for 72 h inhibited mitogen-activated protein kinase (MAPK), nuclear factor-κB (NF-κB) and phosphatidylinositol 3-kinase (PI3K/Akt) signaling, and resulted in a concentration-dependent arrest of cells in the G0/G1 phase of the cell cycle, with alterations in cell cycle-regulatory molecules. Oral administration of PFE (0.1% and 0.2% w/v) to athymic nude mice implanted with A549 cells resulted in significant inhibition of tumor growth. In a recent study, we examined the effect of oral consumption of achievable human doses of PFE on growth, progression, angiogenesis and signaling pathways in two mouse lung tumor protocols. Mice treated with PFE in the drinking water had statistically significantly lower lung tumor multiplicities than mice treated with carcinogens only. PFE treatment caused inhibition of MAPK, NF-κB, PI3K/Akt and mTOR (mammalian target of rapamycin) signaling and markers of cell proliferation and angiogenesis in lungs. Thus, pomegranate may be useful as a chemopreventive/chemotherapeutic agent against lung cancer.

Introduction

Lung cancer is currently the leading cause of cancer mortality in the United States (1). Although surgical resection can be curative for many early-stage cancers, the overall 5-year survival rate for all stages of lung cancer is only 15% (2). In the U.S., smoking contributes to > 90% of all deaths from lung cancer in men and 80% of all deaths from lung cancer in women. Despite surgery with intent to cure, and utilizing the best available therapeutic approaches, < 10% of all lung cancer patients survive 5 years. Thus, prevention is an important part of lung cancer control, especially in high-risk populations, such as current and former smokers. Chemoprevention is defined as the use of specific natural or synthetic chemical agents to reverse, suppress, prevent or delay the carcinogenic process either by blocking the development of early lesions or by inhibiting the progression to invasive cancer. The use of foods and dietary supplements presents a safe chemopreventive strategy.

Pomegranate

Active constituents and studies

Pomegranate (*Punica granatum* L.) is an edible fruit cultivated in Mediterranean countries, Afghanistan, India, China, Japan, Russia and the United States. Edible parts of pomegranate fruit (about 80% of total fruit weight) comprise 80% juice and 20% seed. It is a rich source of crude fibers, pectin, sugars and several tannins, and its juice and seed oil contain certain species of flavonoids and anthocyanins which provide potent antioxidant activity. Pomegranate juice has been reported to exert beneficial effects on the evolution of clinical vascular complications, coronary heart disease and atherogenesis in humans by enhancing endothelial nitric oxide synthase (NOS) bioactivity (3). It has also been shown that the proatherogenic effects induced by perturbed shear stress can be reversed by chronic administration of pomegranate juice (4). Moreover, pomegranate has been reported to suppress NF-κB activation in vascular endothelial cells (5). The hydrolyzable tannins account for 92% of the antioxidant activity of the whole fruit (6).

The first clinical trial to determine the effects of pomegranate juice consumption on prostate-specific antigen (PSA) progression in men with rising PSA levels following primary therapy was reported recently. In this study, the mean PSA doubling time was shown to be significantly increased upon treatment with pomegranate juice in men,

associated with a decrease in cell proliferation and an increase in apoptosis in prostate cancer cells (7).

To determine whether pomegranate has chemopreventive/chemotherapeutic effects against lung cancer, we extracted the edible portion (seed coat and juice) of the fruit and squeezed in 70% acetone-30% distilled water (1:20 v/v). The red extract was filtered, the filtrate was condensed and freeze dried, and the freeze-dried extract was stored at 4°C. This extract, referred to as pomegranate fruit extract (PFE), was analyzed by employing a novel technique of matrix-assisted laser ionization time of flight mass spectrometry (MALDI-TOF MS). PFE was found to contain 6 anthocyanins (pelargonidin 3-glucoside, cyanidin 3-glucoside, delphinidin 3-glucoside, pelargonidin 3,5-diglucoside, cyanidin 3,5-diglucoside and delphinidin 3,5-diglucoside), ellagitannins and hydrolyzable tannins. Studies from our laboratory have shown that PFE possesses remarkable antitumor effects in mouse skin (8) and antiproliferative and proapoptotic effects against prostate cancer (9). PFE was also found to protect against the adverse effects of UVB and UVA radiation by inhibiting cellular pathways in normal human epidermal keratinocytes (10, 11).

Pomegranate and lung cancer

In a recent study, we investigated the antiproliferative effects of PFE on A549 human lung carcinoma cells (12). We first evaluated the effect of PFE on the growth of A549 cells using the MTT assay. We compared the antiproliferative effects of PFE on A549 and normal bronchial epithelial cells (NHBE) cells. PFE treatment (50-150 μg/ml) of A549 cells for 72 h resulted in decreased cell viability, but had minimal effects on NHBE cells at these concentrations. A concentration-dependent growth-inhibitory effect on A549 cells was also confirmed by the trypan blue exclusion assay, which showed a similar growth-inhibitory pattern.

Among the many pathways altered in lung cancer, the most critical involve disruption of normal cell cycle regulation. The deregulation of growth-enhancing and -inhibitory signals can contribute to the alteration of cell cycle control, leading to the proliferation of cancer cells. Lung cancer cells develop the ability to bypass different checkpoints, especially at G1/S and G2/M. An incorrect and/or missing G1/S arrest of the cell cycle causes uncontrolled cellular proliferation. It has been reported that alterations in cyclin/cyclin-dependent kinases (CDKs), which lead to failure of cell cycle arrest, serve as markers of a more malignant phenotype of human lung cancer (13). Our data suggested that PFE treatment (50-150 μg/ml) of A549 human lung carcinoma cells resulted in a concentration-dependent cell cycle arrest in the G1 phase via modulation of CKI-cyclin-CDK machinery. We assessed the effect of PFE on the induction of $p21^{WAF1}$ and $p27^{KIP1}$, which are known to regulate the entry of cells at the G1-S-phase transition checkpoint. We found that PFE treatment of A549 cells caused induction of $p21^{WAF1}$ and $p27^{KIP1}$ in a concentration-dependent manner compared with basal levels. We also assessed the effect of PFE treatment on the protein expression of the cyclins and CDKs, which are known to be regulated by $p21^{WAF1}$. PFE treatment (50-150 μg/ml) of A549 cells resulted in a concentration-dependent decrease in protein expression of cyclins D1, D2 and E, as well as CDK2, 4 and 6 (Fig. 1).

Nuclear factor-κ B (NF-κB) is an ideal target for the development of antiinflammatory agents because it regulates the transcription of various genes that are involved in inflammation, blocks apoptosis and promotes cell proliferation, tumor promotion, angiogenesis and metastasis. NF-κB is present in the cytosol as a heterodimer, usually consisting of its p50 and p65 subunits bound to its inhibitory protein IκB. The NF-κB signaling pathway is reported to play a key role in lung cancer, including resistance to chemotherapy and inducing expression of anti-apoptotic genes. It offers a variety of potential molecular targets for chemopreventive/chemotherapeutic intervention (14).

We found that the NF-κB signaling pathway was highly activated in human lung carcinoma cells. Interestingly, we found that PFE treatment (50-150 μg/ml) significantly inhibited the degradation of IκBα protein and reduced the NF-κB translocation and DNA-binding activity in A549 human lung carcinoma cells (Fig. 1). We further confirmed our results by performing an electrophoretic mobility shift assay (EMSA) and enzyme-linked immunosorbent assay (ELISA). Employing ELISA, we found that

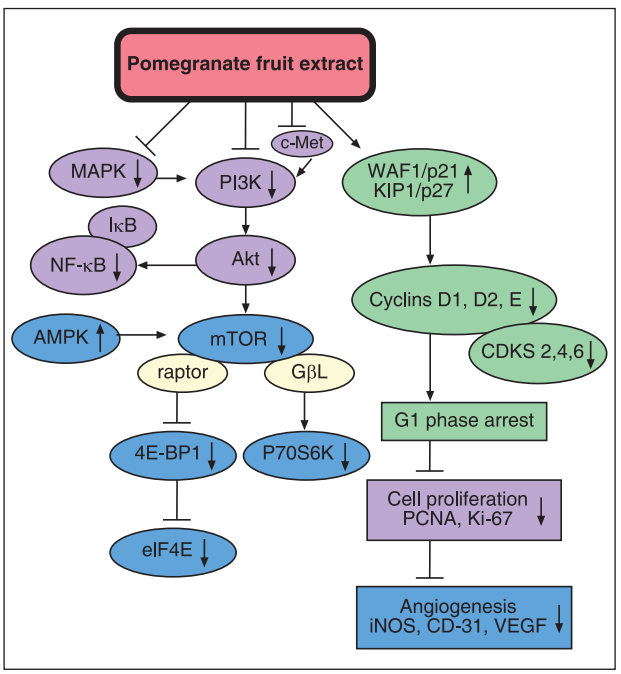

Fig. 1. Effect of pomegranate fruit extract (PFE) on cell signaling pathways, alteration of cell cycle-regulatory molecules, cell cycle arrest and markers of cell proliferation and angiogenesis. *Purple*, denotes molecules for which both *in vivo* and *in vitro* effects of PFE have been shown; *green*, denotes molecules for which only *in vitro* effects of PFE have been shown; and *blue*, denotes molecules for which only *in vivo* effects of PFE have been shown.

pretreatment of A549 cells with PFE (50-150 μg/ml) significantly inhibited nuclear translocation of NF-κB/p65. As shown by EMSA, PFE treatment inhibited NF-κB DNA-binding activity in A549 cells. Immunocytochemical staining of A549 cells further confirmed this effect. In PFE-treated cells, slight cytoplasmic immunostaining was seen with an anti-p65 antibody, whereas control cells showed an intense nuclear fluorescence signifying an inhibitory effect of PFE on the translocation of the NF-κB/p65 subunits into the nuclei.

The mitogen-activated protein kinase (MAPK) family constitutes a superfamily of proteins that include extracellular signal-regulated kinase ERK-1/2, c-Jun *N*-terminal kinase JNK1/2 and p38 kinase (15). Activation of the MAPK pathway occurs in response to integrin-mediated cellular adhesion to the extracellular matrix, which plays a critical role in both tumor metastasis and angiogenesis. The involvement of the MAPK signaling pathway in tumor proliferation is well documented and it is reported to be highly activated during the proliferation of lung epithelial cells and carcinogenesis (16). Therefore, targeting MAPK signaling and interrelated signaling cascades may be critical to preventing lung cancer. We have shown that PFE treatment (50-150 μg/ml) significantly inhibited the phosphorylation of MAPK (ERKl/2, JNK1/2 and p38) proteins in A549 human lung carcinoma cells (Fig. 1).

Phosphatidylinositol 3-kinase (PI3K)/Akt is an important regulatory molecule that is involved in different signaling pathways, as well as in the control of cell growth and malignant transformation. Accumulated evidence indicates that deregulation of the PI3K/Akt pathway occurs frequently in lung cancer, thereby enhancing chemoresistance (17). Interestingly, we found that PFE (50-150 μg/ml) inhibited the expression of both regulatory (p85) and catalytic (p110) subunits of PI3K and inhibited the phosphorylation of Akt at Thr^{308} in A549 human lung carcinoma cells (Fig. 1).

Proliferating cell nuclear antigen (PCNA) and Ki-67 are known cell proliferation markers which are closely associated with the prognosis of certain types of cancers. We found that PFE treatment (50-150 μg/ml) caused a marked reduction in the levels of PCNA and Ki-67, as evidenced by Western blotting and immunocytochemical analysis, respectively, in A549 human lung carcinoma cells, suggesting that PFE possesses a strong antiproliferative potential (Fig. 1).

To establish the relevance of these *in vitro* findings in an *in vivo* situation, athymic nude mice were implanted with human lung carcinoma A549 cells. Twenty-four animals were then randomly divided into 3 groups consisting of 8 animals each. The first group of animals received normal drinking water and served as controls. The animals in groups 2 and 3 received the drinking water supplemented with 0.1% and 0.2% PFE (w/v), respectively. The 0.1% and 0.2% doses of PFE selected were based on the assumption that a typical healthy individual (70 kg) may be persuaded to drink 250 or 500 ml of pomegranate juice extracted from one or two fruits, respectively. The treatment of nude mice with PFE given as the sole source of drinking fluid resulted in inhibition of A549 tumor xenograft growth (Table I). In this protocol, we sacrificed the animals when the tumor reached a volume of 1200 mm^3. In the control group, an average tumor volume of 1200 mm^3 was reached in 55 ± 2 days after tumor cell inoculation. At this time point, the average tumor volumes were 621 and 540 mm^3 in the 0.1% and 0.2% PFE-fed groups, respectively. An average tumor volume of 1200 mm^3 was achieved at 67 ± 4 days after tumor inoculation in the 0.1% PFE group and at 79 ± 3 days after tumor inoculation in the 0.2% PFE group (Table I).

Subsequently, we tested the effect of oral consumption of PFE in two mouse models of lung tumorigenesis (18). Benzo[*a*]pyrene (B[a]P) and *N*-nitroso-tris-chloroethylurea (NTCU) were used to induce lung tumorigenesis in A/J mice. For induction of lung tumorigenesis by B[a]P, 48 A/J mice were randomly distributed into 4 groups of 12 mice each. The mice in group 1 received normal drinking water and were given the vehicle only (cottonseed oil) and served as controls. The animals in groups 2 and 4 received the drinking water supplemented with 0.2% PFE (w/v) until termination of the experiment at day 140. One week after treatment with PFE, mice in groups 3 and 4 were gavaged with a single dose of 20 μmol of B[a]P in 0.2 ml of cottonseed oil. At 84 days post-B[a]P treatment, 6 mice from each group were sacrificed to quantify lung adenomas. At 140 days after B[a]P treatment, the remaining mice were sacrificed and the number of lung adenomas was recorded.

For induction of lung tumorigenesis by NTCU, 48 A/J mice were randomly distributed into 4 groups of 12 mice each. At 48 h before initial treatment, the dorsal skin of the mice was shaved. Mice in group 1 received normal drinking water and were treated with 25 μl of acetone (solvent used for NTCU) and served as controls. Mice in groups 2 and 4 received the drinking water supplemented with 0.2% PFE (w/v) until termination of the experiment

Table I: Effect of oral administration of PFE on A549 tumor growth in athymic nude mice.

Treatment group	Number of days to reach tumor volume	
	600 mm^3	1200 mm^3
Control	35 ± 4	55 ± 2
0.1% PFE	55 ± 3	67 ± 4
0.2% PFE	59 ± 3	79 ± 3

and the water bottles were changed every other day. One week after treatment with PFE, mice in groups 3 and 4 were treated topically with 25 μl of 4 mM NTCU (in acetone) twice a week with a 3-day interval. The animals were sacrificed 240 days after the initial treatment with NTCU.

Treatment with PFE was found to result in a decrease in tumor multiplicity in both B[a]P- and NTCU-treated mice. In the B[a]P + PFE group, the tumor reduction was 53.9% and 61.6% at 84 and 140 days, respectively, as compared to the B[a]P group, and a decrease of 65.9% was seen in the NTCU + PFE group as compared to the NTCU group at 240 days, as shown in Tables II and III, respectively. We found that oral administration of PFE to A/J mice significantly inhibited B[a]P- and NTCU-induced NF-κB and IKKα activation and phosphorylation, and degradation of IκBα protein. Treatment of A/J mice with PFE was found to significantly inhibit B[a]P- and NTCU-induced phosphorylation of the MAPK proteins. PFE treatment of A/J mice resulted in a reduction in the B[a]P- and NTCU-mediated elevated expression of PI3K (p85 and p110) and phosphorylation of Akt (Fig. 1).

Mammalian target of rapamycin (mTOR) is a serine/threonine kinase that belongs to the phosphatidylinositol kinase-related kinase family. It plays a central role in the regulation of cell growth, proliferation and survival, in part by regulating translation initiation. The eIF4E-binding protein-1 (4E-BP1) belongs to a family of repressor proteins that bind to eIF4E and inhibit cap-dependent translation. The binding to eIF4E is determined by the phosphorylation state of 4E-BP1. Hyperphosphorylated 4E-BP1 does not bind to eIF4E. The activated mTOR phosphorylates 4E-BP1, releasing bound eIF4E to bind to eIF4G, stimulating cap-dependent translation. Hence, pharmacological inhibitors of mTOR can sequester eIF4E, consequently inhibiting the translation of factors related to tumor progression. mTOR also phosphorylates and activates p70S6K, which in turn phosphorylates the 40S ribosomal protein S6, leading to the enhancement of translation of mRNAs with a 5'-terminal oligopyrimidine, including mRNAs that encode for ribosomal proteins and elongation factor-1 (EF-1) (19). Overexpression of eIF4E leads to cell transformation or disordered growth (20). It has been reported that activation of either PI3K or Akt, both upstream of mTOR, is sufficient to induce the phosphorylation of both 4EBP1 and p70S6K through mTOR (21), which we also noted in our B[a]P- and NTCU-treated samples. There is also ample evidence that treatment of activated PI3K- or Akt-expressing cells with rapamycin blocks the phosphorylation of p70S6K and 4EBP1, suggesting that mTOR is required for these activities (22). PFE-treated mice showed decreased phosphorylation of mTOR protein expression and its downstream targets, suggesting an effect of PFE on mTOR signaling (Fig. 1).

AMP-activated protein kinase (AMPK) is a metabolic-sensing protein kinase that plays an essential role as an energy sensor, mainly in ATP-deprived conditions. Therefore, AMPK plays a major protective role under metabolic stress conditions. In the activated state, AMPK downregulates several anabolic enzymes and thus shuts

Table II: Inhibitory effect of PFE on B[a]P-induced lung tumors in A/J mice.

Treatment group	No. of mice	At day 84		At day 140	
		Mean tumor multiplicity (mean ± SE)	Tumor incidence (%)	Mean tumor multiplicity (mean ± SE)	Tumor incidence (%)
Control	12	NT	NT	NT	NT
PFE	12	NT	NT	NT	NT
B[a]P	12	7.6 ± 0.52	66.6	13.3 ± 0.39	100
B[a]P + PFE	12	3.5 ± 0.33*	50	5.1 ± 0.17*	83.3

NT = no tumors. *$p < 0.001$ compared with B[a]P-treated group.

Table III: Inhibitory effect of PFE on NTCU-induced lung tumors in A/J mice.

Treatment groups	No. of mice	Mean tumor multiplicity (Mean ± SE)	Tumor incidence (%)
Control	12	NT	NT
PFE	12	NT	NT
NTCU	12	17.6 ± 0.2	100
NTCU + PFE	12	6.0 ± 0.14*	73.0

NT = no tumors. *$p < 0.001$ compared with NTCU-treated group.

down the ATP-consuming metabolic pathways. It has been shown that activation of AMPKα suppresses mTOR signaling by growth factors and amino acids (23). PFE supplementation resulted in increased phosphorylation of the AMPKα protein, whereas B[a]P and NTCU treatment resulted in decreased phosphorylation (Fig. 1).

The c-Met receptor tyrosine kinase and its ligand HGF (hepatocyte growth factor) have been shown to be involved in angiogenesis, cellular motility, growth, invasion and differentiation. c-Met is normally expressed by epithelial cells and has been found to be overexpressed and amplified in a variety of human tumor tissues (24). We found that lungs of B[a]P- and NTCU-treated mice showed increased phosphorylation of c-Met and PFE treatment decreased phosphorylation (Fig. 1).

As mentioned previously, PCNA and Ki-67 are well-known markers of cellular proliferation. The mean percentages of Ki-67 positive cells were 31.1% in the B[a]P-treated group, 16.1% in the B[a]P + PFE group, 28.2% in the NTCU-treated group and 13.1% in the NTCU + PFE group. The mean percentages of PCNA-positive cells were 43.2% in the B[a]P-treated group, 20.5% in the B[a]P + PFE group, 58.5% in the NTCU-treated group and 15.5% in the NTCU + PFE group (Fig. 1).

Inducible nitric oxide synthase (iNOS), platelet-derived endothelial cell adhesion molecule (CD31) and vascular endothelial growth factor (VEGF) are the most common markers of tumor-associated angiogenesis. PFE decreased mean iNOS immunoreactivity scores from 2.7 in the B[a]P-treated group to 0.8 in the B[a]P + PFE group, and from 3.2 in the NTCU-treated group to 1.0 in the NTCU + PFE group. Mean microvessel density was 41.2 microvessels/400X field in the lungs of B[a]P-treated mice compared with 9.1 microvessels/400X field in the lungs of B[a]P + PFE-treated mice; microvessel density was 31.5 microvessels/400X field in the lungs of NTCU-treated mice compared with 11.0 microvessels/400X field in the lungs of NTCU + PFE-treated mice. There was a decrease in the mean VEGF immunoreactivity scores from 2.4 in the B[a]P group to 1.3 in the B[a]P + PFE group, and from 3.0 in the NTCU group to 1.2 in the NTCU + PFE group (Fig. 1).

Conclusions

In summary, we have shown that A549 human lung carcinoma cells were highly sensitive to growth inhibition by PFE in both *in vitro* and *in vivo* experimental models. PFE inhibited G1 phase cell cycle arrest, modulated the CKI-cyclin-CDK network and inhibited MAPK, NF-κB and PI3K/Akt signaling in human lung carcinoma cells. Furthermore, the oral consumption of PFE effectively inhibited lung tumor multiplicity and incidence induced by lung carcinogens. PFE treatment also resulted in downregulation of the activation of MAPK, NF-κB, PI3K/Akt, mTOR signaling and c-Met in lungs of B[a]P- and NTCU-treated mice. PFE reduced the expression of markers of cell proliferation and angiogenesis in the lungs of B[a]P- and NTCU-treated mice. Thus, these findings may be useful for devising strategies for the management of lung cancer. Furthermore, this suggests that PFE inhibited lung tumorigenesis by targeting multiple signaling pathways and associated events, and therefore strongly support its development as a chemopreventive/chemotherapeutic agent against human lung cancer.

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