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**PAPER**

# Cellular and molecular mechanisms of pomegranate juice-induced anti-metastatic effect on prostate cancer cells†

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Prostate cancer is the second leading cause of cancer-related deaths among US males. Pomegranate juice (PJ), a natural product, was shown in a clinical trial to inhibit progression of this disease. However, the underlying mechanisms involved in the anti-progression effects of PJ on prostate cancer remain unclear. Here we show that, in addition to causing cell death of hormone-refractory prostate cancer cells, PJ also increases cell adhesion and decreases cell migration of the cells that do not die. We hypothesized that PJ does so by stimulating the expression and/or activation of molecules that alter the cytoskeleton and the adhesion machinery of prostate cancer cells, resulting in enhanced cell adhesion and reduced cell migration. We took an integrative approach to these studies by using Affimetrix gene arrays to study gene expression, microRNA arrays to study the non-coding RNAs, molecules known to be disregulated in cancer cells, and Luminex Multiplex array assays to study the level of secreted pro-inflammatory cytokines/chemokines. PJ up-regulates genes involved in cell adhesion such as E-cadherin, intercellular adhesion molecule 1 (ICAM-1) and down-regulates genes involved in cell migration such as hyaluranan-mediated motility receptor (HMMR) and type I collagen. In addition, anti-invasive microRNAs such as miR-335, miR-205, miR-200, and miR-126, were up-regulated, whereas pro-invasive microRNA such as miR-21 and miR-373, were down-regulated. Moreover, PJ significantly reduced the level of secreted pro-inflammatory cytokines/chemokines such as IL-6, IL-12p40, IL-1β and RANTES, thereby having the potential to decrease inflammation and its impact on cancer progression. PJ also inhibits the ability of the chemokine SDF1α to chemoattract these cancer cells. SDF1α and its receptor CXCR4 are important in metastasis of cancer cells to the bone. Discovery of the mechanisms by which this enhanced adhesion and reduced migration are accomplished can lead to sophisticated and effective prevention of metastasis in prostate and potentially other cancers.

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## Introduction

Prostate cancer is the second most common cause of cancer-related mortality in US men (13%).[1] Although localized prostate cancer can be successfully treated by surgery and/or radiation, this is not the case for most locally advanced or

## Insight, innovation, integration

The results presented here provide insight into combating prostate cancer by showing the changes in gene expression and cytokine/chemokine levels responsible for the previously reported effects of pomegranate juice (PJ) in deterring prostate cancer progression in humans. Application of the powerful technology of gene, microRNA and protein array analysis showing the upregulation/downregulation of these molecules after PJ treatment of human prostate cancer cells, paves the way to unraveling the mechanisms involved in the beneficial effects of PJ on prostate cancer patients. Therefore, integration of technology and biology in this study has enabled us to rapidly establish the basis to understand the effects of PJ on metastasis of one of the most deadly diseases of males in the US.


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widespread disease. Initially, these prostate cancer situations can be controlled with hormone ablation therapy, taking advantage of the growth dependence of prostate cancer on testosterone. However, over time, the cancer develops ways to bypass dependence on circulating hormone (androgen), becoming castration resistant prostate cancer (CRPC) that is highly aggressive and metastatic.[2] Chemotherapy has been available for a number of years to treat hormone refractory prostate cancer but such treatment is usually reserved for the more advanced stages of tumor progression when the cancer is already metastasized to the bone. This is so because prostate cancer cells don't grow rapidly until they reach the bone marrow and hence do not respond as well as other cancers to the chemotherapeutic agents. This, coupled with the very serious side effects of chemotherapy, makes it the treatment of choice primarily for advanced cancers.[3]

As a result, researchers have turned to developing novel strategies to fight prostate cancer. One type of treatment involves developing immunotherapies such as vaccines against PSA (prostate specific antigen), a protein specifically produced by prostate cells, both normal and cancerous. FDA approved therapeutic cancer vaccine Sipuleucel-T (Provenge®) has been shown to considerably improve the overall survival of some patients, but with a median survival rate of only 4.1 months. Moreover, there was no effect on time of progression in patients who were asymptomatic or with limited metastatic disease and treatment is costly.[4] Nevertheless, this vaccine is important because it represents a prototype that can be used to develop other cancer vaccines. PROSTVAC-VF, a PSA-based vaccine that uses a small pox-related virus has also been tested in men with metastatic disease and was shown to have an effect on survival.[5] These findings indicate that immunotherapy is promising.

Another very promising therapy is related to the development of novel anti-androgen agents. For example, abiraterone, an irreversible inhibitor of CYP17A1 lyase (an enzyme critical for synthesis of androgen in the testes, adrenal glands and prostate tissue), has proved to be effective in improving survival.[6] Furthermore, androgen receptor (AR) antagonist MDV3100 is a very potent inhibitor of AR; it binds to this receptor with a much higher affinity than bicalutamide (Casodex), the currently prescribed androgen receptor inhibitor.[7]

Recently, there has been a renewed push to identify natural remedies to fight prostate cancer. Among the latter is pomegranate juice (PJ). In a phase II clinical trial performed at the University of California Los Angeles (UCLA), patients with rising PSA were given 8 ounces of pomegranate juice by mouth daily until disease progression. PSA doubling time significantly increased with treatment from a mean of 15 months at baseline to 54 months post-treatment ($p < 0.001$).[8] Although extremely promising, a major drawback of this study was the absence of a proper placebo control. Nevertheless, statistically significant prolongation of PSA doubling time suggested a potential of pomegranate juice for treatment of human prostate cancer.

As a result of these findings in humans, several studies have shown that PJ has anti-proliferative and pro-apoptotic effects[9] and that those effects occur through modulation of cyclin kinase inhibitor.[10] One study using the Matrigel invasion assay showed the capability of PJ to significantly reduce the invasiveness of prostate cancer cells *in vitro*.[11] The latter investigators showed that pomegranate juice decreases the levels of phospholipase A2 ($PLA_2$), which is the enzyme responsible for prostaglandin production in the arachidonic acid (AA) metabolic pathway.[12] Inhibitors of $PLA_2$ are able to reduce the production of matrix metalloproteinase 2 (MMP-2) by prostate cancer cells.[13] Numerous studies support a critical role for MMPs in tumor metastasis.[14] In addition, one component of PJ was shown to stimulate the expression of E-cadherin,[15] a molecule that is important in adhesion of epithelial cells.[16]

Despite these findings, very little is known about the cellular and molecular mechanisms affected by PJ that deter cancer progression, in particular in processes leading to metastasis. We hypothesized that PJ inhibits the migratory and metastatic properties of hormone refractory prostate cancer cells by stimulating the expression and/or activation of molecules that alter the cytoskeleton and the adhesion machinery of the cell, resulting in enhanced cell adhesion and reduced cell migration. To test this possibility we have used prostate cancer cell lines that are hormone refractory and invasive (DU145 and PC3) and applied an integrative approach to discovering the genes, miRNAs and proteins involved in PJ inhibition of prostate cancer progression to uncover pathways towards mechanistic understanding and more sophisticated and effective treatments of the disease.

## Materials and methods

### Materials

DU145 and LNCaP prostate cancer epithelial cell lines were purchased from ATCC (Manassas, VA). PC3 prostate epithelial cell line was a gift from A. Walker (UC Riverside). pcDNA 3.1 HMMR vector was a gift from E. Turley (University of Western Ontario). RPMI 1640 media was acquired from Mediatech (Manassas, VA) and fetal bovine serum from Sigma Aldrich (St. Louis, MO). Stromal derived factor 1 alpha (SDF1α) was obtained from ProSpec (Boca Raton, FL). Human Genome U133Plus 2.0 Arrays were purchased from Affymetrix (Santa Clara, CA) and the RNeasy RNA Isolation Kit for RNA preparation from Qiagen Inc. (Valencia, CA). The Luminex Multiplex assays were obtained from Millipore (Billerica, MA). E-cadherin Ab was from Cell signaling (Danvers, MA). ICAM-1 Ab and tropomyosin 1 Ab were purchased from Developmental Studies Hybridoma Bank (Iowa city, Iowa). Secondary Ab was from Thermo Scientific (Rockford, IL). Detection was done using Supersignal West Dura kit from Thermo Scientific (Rockford, IL). Transfection reagents Lipofectamin™ 2000 were purchased from Invitrogen (Carlsbad, CA). AposcreenTM Annexin V-FITC kit from Southern Biotech (Birmingham, AL). All oligonucleotide primers for qPCR were obtained from IDT (Coralville, IA) and the qPCR iQ SYBR green supermix kit from Bio-Rad (Hercules, CA). CXCR4 specific inhibitor AMD3100 was obtained from Sigma Aldrich (St. Louis, MO). Synthesized RNA duplexes of microRNA mimics and microRNA inhibitors were purchased from Thermo Scientific (Rockford, IL). E-cadherin siRNA was purchased from Abnova

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(Taiwan, China). The pomegranate juice was purchased from POMx Wonderful.

### Cell culture

DU145 and PC3 are hormone-independent prostate cancer epithelial cell lines. LNCaP is an androgen-responsive prostate cancer epithelial cell line. Cells were cultured at 37 °C with 5% $CO_2$ in RPMI 1640 supplemented with 10% fetal bovine serum, penicillin (100 I.U $ml^{-1}$L) and streptomycin (100 μg $ml^{-1}$) and used at the times indicated in the results.

### Adhesion assay

$3 \times 10^5$ DU145, PC3 and/or LNCaP cells were plated on gelatin-coated 6-well plates (B&D Biosciences), allowed to adhere and 24 h later treated with 1% and/or 5% PJ for 12 h and/or 24 h. Cells were then trypsinized and the time required to detach all cells was recorded as an indicator of cell adhesiveness. The juice was sterilized by filtration. The filtrate was then centrifuged at 13 000 rpm for 5 min, the supernatant collected to remove any particulate matter and then frozen in small aliquots to reduce the numbers of freeze-thaw cycles that can potentially damage the contents of the juice.

### Migration assay

Confluent DU145 and PC3 cells were scratch wounded using a rubber scraper, washed and treated with 1% or 5% PJ. Cell migration was determined by measuring the distance migrated by the cells from the wounded edge to the leading edge of migration at 12 h, 24 h, 48 h and 72 h after treatments were initiated. Scraped cells without treatment were used as controls.

### Chemotaxis assay

The upper side of 8 μm pore size polycarbonate membranes of transwells (BD Biosciences, San Jose, CA) were coated with 50 ng $ml^{-1}$ type I collagen (Sigma Chemical Co.). DU145, PC3 and/or LNCaP cells ($1\times10^5$) in 100 μl culture medium were plated on the upper side of transwell membranes and were allowed to adhere for 4 h. Then the wells were introduced into 24-well plates and 1000 μl RMPI 1640 with 10% FBS medium was added to lower chamber. Cells were treated with 1% and/or 5% PJ for 12 h. SDF1α (100 ng $ml^{-1}$) was added to the lower chamber and the cells were allowed to migrate for 3.5 h at 37 °C. The cells on the side of the membrane facing the upper chamber were removed with a cotton swab, and the membranes were then fixed and stained with 2% toluidine blue in 4% paraformaldehyde. Cells were counted in 8 high power fields (HPF)/filter to obtain the average number of cells per field.

### Total RNA extraction

DU145 cells were treated with PJ for 12 h and total RNA was extracted using the RNeasy RNA isolation Kit according to manufacturer's protocol. Briefly, cells were washed with ice-cold 1X PBS, and lysed on ice with lysis buffer. Cell lysates were then spun at 12 000 rpm for 5 min to remove cell debris, followed by organic extraction to remove proteins. Then lysates were loaded into isolation columns and the final RNA product was dissolved in nuclease-free water. RNA quality was assessed on the Agilent Bioanalyzer 2100 using the Agilent RNA 6000 Nano Assay kit (Agilent Technologies, Waldbronn, Germany) and the concentration determined using the NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Inc., Wilmington, DE USA).

### Affymetrix microarray and data analysis

Affymetrix Human Genome U133 Plus 2.0 Arrays, which contain more than 54 000 probe sets representing approximately 38 500 genes and gene sequences, were used. Cells were treated with 5% PJ for 12 h and total RNA was extracted and evaluated as described above. Capillary electrophoresis using an Agilent Bioanalyzer 2100 to confirm the RNA quality levels was used before performing the array assays. RNA from cells without treatment was used as control. A single $\log_2$ expression measure for each probe set was calculated from image files (CEL format) using the robust multi-array analysis (RMA) procedure using Agilent GeneSpring GX software. The changes of expression level between untreated and specific PJ components-treated samples were compared. Only genes that were over- or under-expressed by $>2$-fold were considered. Once we identified genes of potential interest, we verified their increase or decrease in expression by RT-qPCR.

### Real time quantitative PCR

1 μg RNA was reverse-transcribed to cDNA by RETROscript Reverse Transcription Kit (Ambion) at 44 °C for 1 h and 92 °C for 10 min. 2 μl of cDNA from the reverse transcription reaction were added to 23 μl real-time quantitative PCR mixture containing 12.5 μl 2 × SYBR Green SuperMix (Bio-Rad) and 200 nM oligonucleotide primers. PCRs were carried out in a Bio-Rad MyiQ5 Real-Time PCR Detection System (Bio-Rad). The thermal profile was 95 °C for 3 min followed by 40 amplification cycles, consisting of denaturation at 95 °C for 10 s, annealing at 60 °C for 30 s. Fluorescence was measured and used for quantitative purposes. At the end of the amplification period, melting curve analysis was done to confirm the specificity of the amplicon. Fold changes of genes after treatment with PJ were calculated by the Pfaffl method to normalize the Ct values to the *GADPH* internal control. The following primer sequences were designed with IDT PrimerQuest (http://www.idtdna.com/Scitools/Applications/Primerquest/) and used for the reactions:

***GADPH***, TCGACAGTCAGCCGCATCTTCTTT and ACCAAATCCGTTGACTCCGACCTT;

***MARCKS***, TTGTTGAAGAAGCCAGCATGGGTG and TTACCTTCACGTGGCCATTCTCCT;

***ICAM-1***, ATAACCGCCAGCGGAAGATCAAGA and CGTGGCTTGTGTGTTCGGTTTCAT;

***CLDN1***, ATGGAAAGGGTGTTGGCATTGGTG and CACTTGGGTGTTTGAGCATTGCCT;

***HMMR***, ATTCAGTTGTCGAGGAGTGCCAGT and AGTGCAGCATTTAGCCTTGCTTCC;

***COL1A1***, CAATGCTGCCCTTTCTGCTCCTTT and CACTTGGGTGTTTGAGCATTGCCT;

***CHN1***, TGAAACTACTGCCACCTGCTCACT and TGGGTCCAAAGACGATTCCAAGGT;

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***PRCKE***, CAACCAAGCAAGCTCTAACCGCAA and TTGTCCTGTAGGAAAGGCCCAGTT;
***NEXN***, TCAGCCCAAGACCACATAGAGCAA and TCTTTCTTCCCTGGCTCTCTGCAT;
***ANLN***, AGCTCACTCTTCTCACCAATGCCA and AAGCGGTACCAGGCTGTTCTTGTA.

### MicroRNA arrays and data analysis

DU145 cells were treated with 5% pomegranate juice for 12 h with the same conditions used in the Affymetrix microarray. Total RNA was collected with RNeasy RNA Isolation Kit for microRNA arrays. RNA from cells without treatment was used as control. We then utilized SYBR based real-time PCR to quantify mature microRNA expression (Quantobio Technology). *E. coli* polyA polymerase was used to add an adenine tail at the 3′ end of RNA molecules that lack a polyA tail. Following the oligodT annealing, a universal tag was attached to the 3′ end of cDNAs during the cDNA synthesis using retro-transcriptase superscript III (Invitrogen). With this universal tag, qPCR was performed with micro-RNA-specific forward primer and a reverse universal primer mix.

### miRNA mimics, inhibitor, siRNA and vector transfection

DU145 and/or PC3 cells (80–90% confluent) were transfected with Lipofectamine 2000 (Invitrogen) following the manufacture's protocols. 60 nM miR-21 miRIDIAN mimics, miR-335 miRIDIAN hairpin inhibitor (Thermo Scientific), 40 nM E-cadherin chimera siRNA (Abnova) and 8 μg $ml^{-1}$ pcDNA3.1 HMMR vectors were transfected. Scratch wound assay was performed as described above, 24 h after transfection.

### Immunoblotting

DU145 cells were treated with 5% PJ for 12 h, washed with ice cold 1× PBS, and lysed on ice with lysis buffer containing 0.5% Triton X100, 0.5% Nonidet P-40, 10 mM Tris, pH 7.5, 2.5 mM KCl, 150 mM NaCl, 30 mM b-glycerophosphate, 50 mM NaF, 1 mM $Na_3VO_4$, 0.1% SDS and additional protease inhibitor cocktails (Sigma). Protein concentrations were measured using the DC protein assay kit (Bio-Rad). Equal amounts of protein in the cell extracts were mixed with sample buffer, boiled, and analyzed using 10% acrylamide SDS-PAGE. Immunoblotting was performed with the E-cadherin polyclonal Ab (Cell signaling), ICAM-1 polyclonal Ab, tropomyosin 1 polyclonal Ab (Hybridoma Bank) and the HRP-conjugated secondary Ab (Thermo Scientific), followed by incubation with West Dura extended-duration substrate (Thermo Scientific). Blots were then reprobed for histone 2A or GAPDH antibody to show equal loading of proteins.

### Statistical analysis

Data analysis was performed using the one-way ANOVA on raw data using GraphPad Instat software (GraphPad Software Inc.).

## Results

### Effect of pomegranate juice on growth, adhesion, migration and chemotaxis of prostate cancer cells

To set up and standardize the cell culture system to test the effects of PJ on hormone-refractory prostate cancer cell function, we used DU145 and PC3, which are two invasive hormone-independent prostate cancer cell lines. We treated these cells with 1% and 5% filtered pomegranate juice and measured the effect of the juice on cell growth over time (Fig. 1). Although at 12 h there was no measurable effect of the juice on cell growth, by 24 h it was clear that the 5% concentration was not allowing the cells to grow and by 48 h the cultures contained many floating cells, indicating that this PJ concentration caused the cells to die. In contrast, the cultures treated with 1% PJ continued to grow, although they showed some floating cells. Untreated cells (control) showed no signs of cell death. By 72 h, the cultures treated with 5% PJ still contained some cells that were attached to the dish but most of the cells were in suspension (Fig. 1A and B).

Despite the fact that many cells died with the treatments, especially with 5% treatment, in both sets of treated cultures there were living cells at the end of the experiment. We found that the remaining cells strongly adhered to the gelatin-coated substrate, as indicted by increased time to detach all cells from

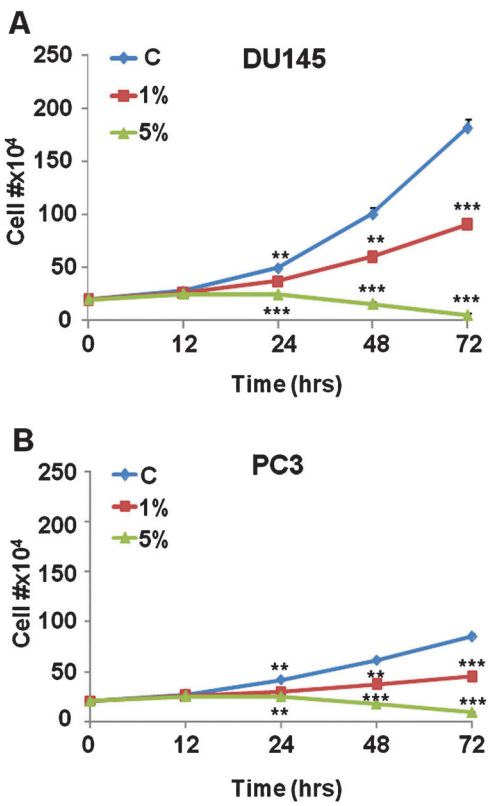


**Fig. 1 Pomegranate juice inhibits growth of hormone-independent prostate cancer cells.** DU145 (A) and PC3 (B) prostate cancer cells were treated with 1% and 5% PJ and counted for increasing times after initiation of treatment. Controls represent no PJ. Media containing PJ was changed daily. Bars represent standard error of the mean. ***$p < 0.001$; **$p < 0.01$. Repeated many times.

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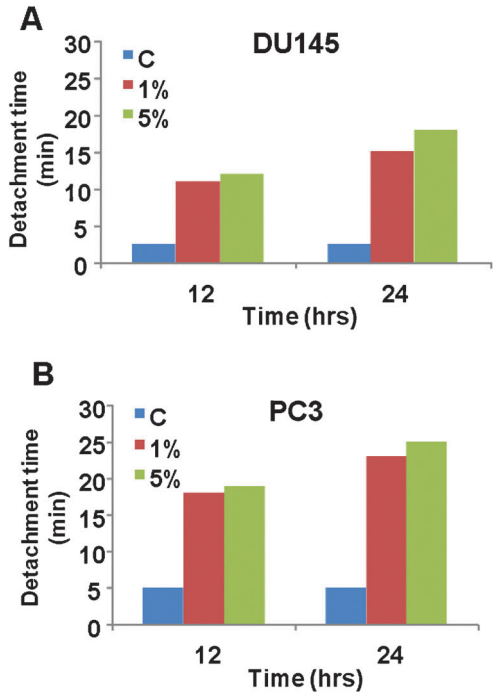


**Fig. 2 Pomegranate juice stimulates hormone-independent prostate cancer cell adhesion**. DU145 (A) and PC3 (B) cancer cells were plated on gelatin-coated dishes and 24 h later media was changed and the cells were treated with 1% or 5% pomegranate juice. We tested for adhesion to the substrate at 12 and 24 h after initiation of treatment by recording the time it took for trypsinization to remove all of the cells from the dish. Control represents no pomegranate juice. Within each experiment, the times of trypsinization were the same within 1 min for each specific treatment. Repeated many times.

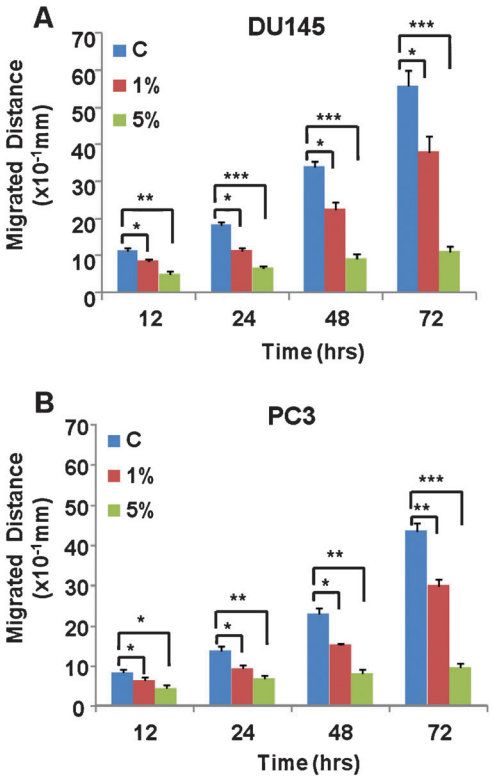


**Fig. 3 Pomegranate juice inhibits hormone-independent prostate cancer cell migration.** DU145 (A) or PC3 (B) prostate cancer cells were treated with 1%, and 5% PJ for 72 h and the distance migrated by the cells from the wounded edge to the leading edge was measured at the indicated time points. Controls represent no PJ. Media and PJ were changed daily. Bars represent standard error of the mean. ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$. Repeated at least 3 times.

the culture dish in comparison with untreated cells; by 12 h of PJ treatment, the treated cultures required 4–5 times longer to be released by trypsinization and by 24 h the time to release further increased (Fig. 2). At both time points the cells all still looked healthy and no floating cells were seen in any of the cultures.

The PJ-induced increase in adhesion of the cells that did not die suggested that this juice also might affect cell migration. Indeed, using the scratch wound assay, we found that both 1% and 5% pomegranate juice inhibited the migratory capabilities of these cells. We measured the distance that cells migrated from the wounded edge to the migration front and found it significantly reduced in the treated cells beginning as early as 12 h after treatment. This pattern of delayed migration continued over time (Fig. 3).

Because the chemokine stromal-derived growth factor one alpha (SDF1α) is very important in attracting cancer cells to the bone marrow,[17] we tested the possibility that PJ would inhibit chemotaxis towards SDF1α. We first tested whether the cancer cells respond to SDF1α and found that this chemokine chemoattracts these cells (Fig. 4C and D). This chemoattraction is specific because the specific inhibitor of its receptor, CXCR4, completely obliterates its effect on both cell types. When the cells were pre-treated with PJ for 12 h prior to initiation of the chemotaxis assay towards SDF1α, we found that the juice significantly inhibited chemotaxis at both concentrations (Fig. 4A and B). Therefore, the fluid fraction of PJ has the potential to inhibit metastasis of these cells to bone marrow.

To determine whether these effects of PJ are also found in androgen-sensitive cells, we used LNCaP prostate cancer cells and performed similar studies using the adhesion and chemotaxis to SDF1α assays. LNCaP cells are less sensitive to PJ-induced increase in adhesion because we see only approximately doubling of the release time for the cells from the substrate as with either DU145 or PC3 cells (Fig. 5A). In contrast, LNCaP cells are considerably more sensitive to PJ inhibition of SDF1α-induced chemotaxis (Fig. 5B).

### Effect of pomegranate juice on the expression of genes involved in functions of the cytoskeleton and in cell adhesion

To understand how PJ inhibits these critical cellular processes involved in metastasis, we used an integrative approach to examine the effects of the juice on gene and miRNA expression and on production of pro-inflammatory cytokines. To study the effect of PJ on cell adhesion and migration related genes, total RNA obtained from DU145 treated with 5% of the aqueous faction of PJ for 12 h was analyzed for gene expression using Affymetrix U133 Plus 2.0 microarrays. This time point and this concentration were chosen because under these conditions changes in cell function are already occurring but

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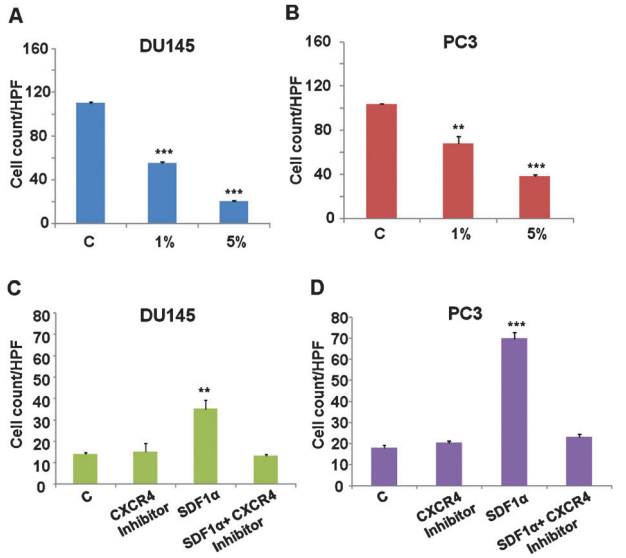


**Fig. 4 Pomegranate juice inhibits hormone-independent prostate cancer cell chemotaxis to SDF1α.** (A,B) DU145 and PC3 cancer cells were allowed to attach to the top of the filter of the chemotaxis chamber for 4 h and then treated with 1% or 5% PJ for 12 h. At this time, 100 ng ml$^{-1}$ of SDF1α were introduced into the lower chamber and the cells found on the bottom of the filter counted 3.5 h later. Control had no PJ treatment. (C,D) DU145 and PC3 cancer cells were pre-treated with 5 μg ml$^{-1}$ of a specific CXCR4 inhibitor for 15 min before introducing 100 ng ml$^{-1}$ of SDF1α into the lower chamber. The number of cells found on the underside of the filter was counted 3.5 h later. Negative controls consist of treatment with inhibitor alone. Bars represent standard error of the mean. ***$p < 0.001$; **$p < 0.01$. Repeated at least 3 times.

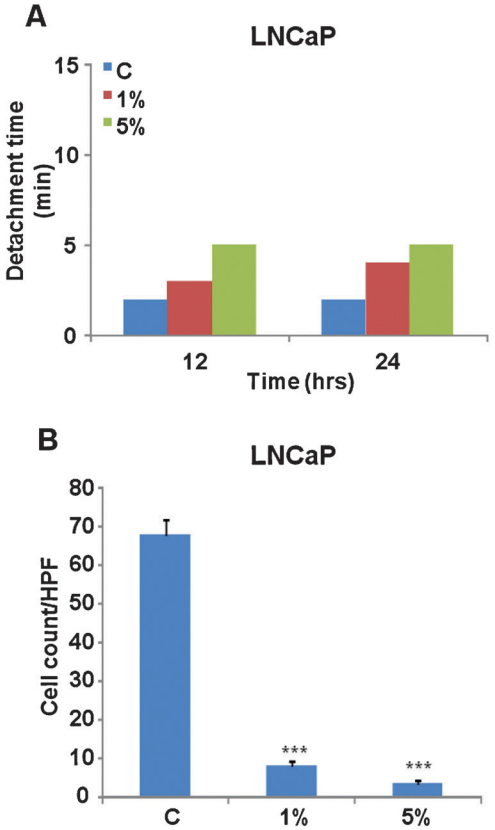


**Fig. 5 Pomegranate juice stimulates cell adhesion and inhibits chemotaxis to SDF1α of LNCaP prostate cancer cells.** (A) LNCaP cancer cells were plated on gelatin-coated dishes and 24 h later media was changed and the cells were treated with 1% or 5% pomegranate juice. We tested for adhesion to the substrate at 12 and 24 h after initiation of treatment by recording the time it took for trypsinization to remove all of the cells from the dish. Control represents no PJ treatment. Within each experiment, the times of trypsinization were within 1 min of each other for each specific treatment. (B) LNCaP cancer cells were allowed to attach to the top of the filter of the chemotaxis chamber for 4 h and then treated with 1% or 5% PJ for 12 h. At this time, 100 ng ml$^{-1}$ of SDF1α were introduced into the lower chamber and the cells found on the bottom of the filter counted 3.5 h later. Control had no PJ treatment. Bars represent standard error of the mean. ***$p < 0.001$.

no cell death is seen, as shown in Fig. 1 and 2. This was important because we want to determine what genes are modulated by PJ before some of the cells became apoptotic. The data show that PJ stimulates the expression of several genes involved in cell adhesion and inhibits the expression of several genes involved in cytoskeleton function and in cell migration (Fig. 6). The genes we show that are increased significantly all enhance adhesion or are tumor suppressors. Those that are decreased significantly all are related to migration or to cytoskeletal elements or ECM molecules that facilitate migration.

To verify the effects of PJ on gene expression shown by Affymetrix mRNA arrays, we examined the level of some of the genes that function in cell adhesion and in cell migration. For cell adhesion, we looked at intercellular adhesion molecule 1 (*ICAM-1*), myristolytated alanine-rich protein kinase C substrate (*MARCKS*), claudin 1 (*CLDN1*) at the mRNA level using RT-PCR, and E-cadherin, ICAM-1 and tropomyosin at the protein level using western blots. For migration we examined hyaluranan-mediated motility receptor/CD168 (*HMMR*), collagen type I alpha1 (*COL1A1*), protein kinase C epsilon (*PRCKE*), anillin (*ANLN*), nexilin (*NEXN*), N-chimearin (*CHN1*). For the RT-PCR, total RNA was extracted from DU145 cells treated with 5% PJ for 12 h (Fig. 7A). The mRNA fold change of these genes was highly consistent with our Affymetrix array results. For the protein levels we used antibodies specific for E-cadherin and ICAM-1 and found them to be increased by 5% PJ after 24 h treatment, confirming the array result (Fig. 7B and C). Tropomyosin was predicted to be elevated (miRNA21 was down-regulated, see Fig. 9) and we show that this is the case (Fig. 7D).

To determine mechanistically whether the PJ effects on cell migration can be reversed, we chose to inhibit E-cadherin because loss of this adhesion protein is critical for invasion of epithelial tumor cells, and to over-express HMMR, the gene that showed the highest decrease in our gene array. We find that the inhibitory effect of PJ on cell migration is partially reversed by E-cadherin siRNA and HMMR overexpression (Fig. 8). Scratch wounds on DU145 and PC3 cells were made 24 h after E-cadherin siRNA and HMMR vector transfection and the migrated distances were measured 36 h later with and/or without 1%PJ treatments. We find that PJ treatment significantly decreases cell migration of untransfected cells but

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List of selected gene expression changes with POMEGRANATE JUICE treatment

| Gene Name | Product | Fold-change vs.Control | Function |
|---|---|---|---|
| ICAM-1 | Intercellular adhesion molecule 1 | ↑3.3 | Cell adhesion |
| MARCKS | Myristoylated alanine-rich protein kinase C substrate | ↑2.8 | Cell adhesion |
| PDCD4 | Programmed cell death 4 | ↑2.4 | Tumor suppressor |
| CDH1 | E-cadherin | ↑2.4 | Cell adhesion |
| CLDN1 | Claudin 1 | ↑2.3 | Cell adhesion |
| HMMR | hyaluronan-mediated motility receptor (CD168) | ↓6.7 | Hyaluronan receptor, cell migration |
| ANLN | Anillin | ↓2.8 | Actin binding |
| COL1A1 | Collagen type I α1 | ↓2.7 | ECM component |
| PRKCE | Protein kinase C, epsilon | ↓2.4 | Signal transduction |
| NEXN | Nexilin | ↓2.3 | Actin binding |
| CHN1 | N-chimearin | ↓2.1 | Cell motility |

**Fig. 6 Pomegranate juice changes the expression profile of genes involved in the cytoskeleton and cell adhesion machinery.** RNA was extracted from DU145 cells that had been treated with 5% PJ for 12 h and Affymetrix array analysis was performed as described in Materials and methods. Relative mRNA levels are presented as fold change compared with untreated controls. Only genes with fold change greater than 2 are depicted. Red line separates genes increased from genes decreased.

there is no significant difference between cell migration of untreated cells and E-cadherin siRNA (Fig. 8A and B) and HMMR vector (Fig. 8C) transfected cells. These results indicate that the effects of PJ on cell migration are mediated, at least in part, through increasing E-cadherin and decreasing HMMR in DU145 cells and through increasing E-cadherin in PC3 cells.

### Effect of pomegranate juice on the level of microRNAs related to cell adhesion and migration

MicroRNAs (miRNAs) are naturally-occurring small non-coding RNAs that function as negative regulators of gene expression. They regulate important cellular functions such as cell proliferation, apoptosis, differentiation and development.[18] Mature miRNAs bind to target mRNAs which subsequently results in either direct cleavage of the targeted mRNAs or inhibition of translation. To determine the effects of PJ on adhesion and migration-related miRNAs, we used miRNA arrays to analyze RNA obtained from DU145 cells treated with 5% PJ for 12 h (Fig. 9). We found that miRNAs that regulate the genes we identified as elevated were decreased (miRNA-21 and miRNA-373) whereas those that regulate genes that were decreased show increased expression (miRNA-335 and miRNA-205). An apparent exception to this pattern is the increase in miRNA-200 which regulates E-cadherin, a gene that we identified as having increased expression. This is because miRNA-200 inhibits a repressor for the E-cadherin gene, hence the increase of miRNA-200 is consistent with the pattern of the other miRNAs.

To demonstrate mechanistically if levels of expression of these miRNAs correlate with function, we chose to treat the cells with the mimics for miRNA-21 (because this miRNA is considered to be proinvasive) and to inhibit miRNA-335 (because this miRNA was the most over-expressed). Both of these treatments reversed the ability of PJ to inhibit cell migration of both DU145 and PC3 cells (Fig. 10A–D).

### Effect of pomegranate juice on the level of selected cytokines and chemokines

Many proinflammatory cytokines and chemokines contribute to cancer progression. To determine the effect of PJ on production of some of these proteins, we analyzed the media collected from DU145 and PC3 cells treated with 1% or 5% PJ for 18 h, by using Luminex Multiplex Array assays. We tested for the levels of the following pro-inflammatory proteins IFN-γ, IL-1α, IL-1β, IL-2, IL-4, IL-6, IL-10, IL-12p40, IL-12p70, IL-13, IL-17, TNF-α IP-10, and CCL5/RANTES. In both cell lines PJ decreased the levels of IL-6 and IL-12p40 (Fig. 11A and B) production. In addition, DU145 cells showed decrease in IL-1β (Fig. 11C), and PC3 cells showed marked decrease in CCL5/RANTES (Fig. 11D).

## Discussion

The studies presented here delineate potential cellular and molecular mechanisms involved in the anti-metastatic effect of PJ on prostate cancer cells. We show that the fluid phase of the juice: (i) stimulates two prostate cancer cell lines to adhere strongly to the substrate; (ii) inhibits the migratory capabilities of these cells and chemotaxis toward SDF1α; (iii) stimulates expression of genes involved in cell adhesion while reducing expression of genes involved in cytoskeletal functions and in cell migration; (iv) increases the levels of adhesion-enhancing miRNAs while reducing the level of pro-invasive miRNAs; (v) reduces the level of the pro-inflammatory cytokines/chemokines IL-6, IL-12p40, IL-1β and RANTES. The results of this integrative approach are summarized in Fig. 12.

During progression of prostate cancer, the epithelial cells of the prostate undergo hyperplasia, followed by prostatic intraepithelial neoplasm, which then develops into invasive adenocarcinoma that finally becomes metastatic leading to spread of the cancer cells primarily to the lymph nodes, bone marrow and lung. Metastatic cells undergo a transition from stationary to migratory. This transition involves loss of adhesion and rearrangement of cytoskeletal elements that allow the cells to move. Our results show that PJ deters that movement by increasing cell adhesion molecules and decreasing molecules that facilitate cell migration. By using an integrative approach to detect gene, miRNA and proinflammatory protein changes induced by the juice, we

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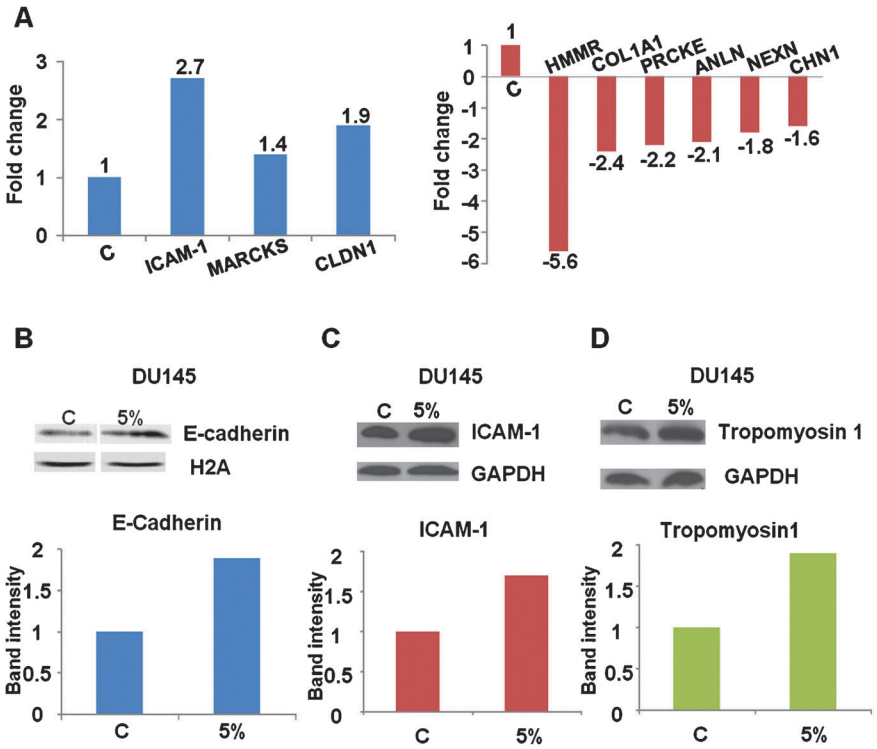


**Fig. 7 Verification of the effect of pomegranate juice on gene expression using RT-qPCR.** (A) The mRNA level of *ICAM-1*, *MARCKS*, *CLDN1*, *HMMR*, *COL1A1*, *PRCKE*, *ANLN*, *NEXN* and *CHN1*, were determined by using qPCR with RNA extracted from DU145 cells treated with 5% PJ for 12 h. (B–D) Immunoblot analysis for E-cadherin, ICAM-1 and tropomyosin 1 with protein extracts from DU145 cancer cells treated with 5% pomegranate juice for 24 h. Performed one time because the results supported the data from the array

have developed a model that may explain how PJ inhibits progression of prostate cancer to metastasis.

***The gene microarray results*** show that expression of genes involved in the cell adhesion machinery are stimulated by PJ treatment whereas genes that stimulate migration are down-regulated. For example, E-cadherin and claudin 1 are important components of tight junction protein complexes that keep epithelial cells together; in this case the epithelial cells of the prostate where the cancer develops. Intercellular adhesion molecule-1 (ICAM-1) is also important in stabilizing cell–cell interactions. Therefore, PJ might increase cell adhesion through up-regulation of these cell junction proteins preventing the cells from breaking away from the adenocarcinoma. PJ also up-regulates myristoylated alanine-rich protein kinase C substrate (MARCKS) which is an actin-binding protein that normally associates with the plasma membrane where vinculin and talin are present in focal adhesions.[19] Phosphorylation of MARCKS by protein kinase C inhibits its association with the plasma membrane leading to movement of the protein into the cytosol, resulting in cell spreading onto the substratum.[20] MARCKS also has been shown to play a role in cell adhesion and cell motility through regulation of the actin cytoskeletal structure.[21] Our gene microarray results also show that PJ down-regulates the expression of genes related to cell migration. HMMR functions as a hyaluronan (HA) receptor and the binding of HA to HMMR can stimulate the RhoA-activated protein kinase (ROCK) signal transduction pathway, leading to tumor cell migration and invasion in various cancers.[22] In addition, PJ down-regulates actin binding proteins anillin and nexillin which are involved in regulation of the structure of the cytoskeleton.[23]

***MicroRNAs* (*miRNAs*)** have been shown to have profound impact on post-transcriptional gene regulation. Aberrant expression of miRNAs occurs in diverse types of human cancer and in different stages of disease progression.[24] miRNA-335 has been identified as a metastasis-suppressive miRNA in breast cancer by inhibiting type I collagen (COLA1) and tenascin C (TNC).[25] Type I collagen is an extracellular matrix molecule involved in cytoskeletal control and tenascin C is involved in the regulation of cell migration.[26] PJ might inhibit the expression of COL1A1 and TNC in prostate cancer cells through up-regulation of the metastasis-suppressive miRNA-335. miRNA-205 has been shown to reduce cell invasion in prostate cancer by inhibiting PKCε (PRKCE) and N-chimearin (CHN1).[27] PKCε enhances migration and invasion and promotes autocrine cell-signaling events in prostate cancer cells.[28] N-chimaerin is a GTPase-activating protein that, if down-regulated, can give rise to the loss of filopodia and reduction of cell migration.[29] PJ could inhibit the expression of PRKCE and CHN1 in prostate cancer cells through up-regulating miRNA205, which would in turn lead to reduced cell migration and invasion. The miRNA-200 family has been shown to inhibit ZEB1 and ZEB2, which are transcriptional repressors of the E-cadherin gene.[30] Loss of E-cadherin is one of the molecular hallmarks driving epithelial-mesenchymal transition that is an important process to initiate metastasis.[31] PJ might stimulate the

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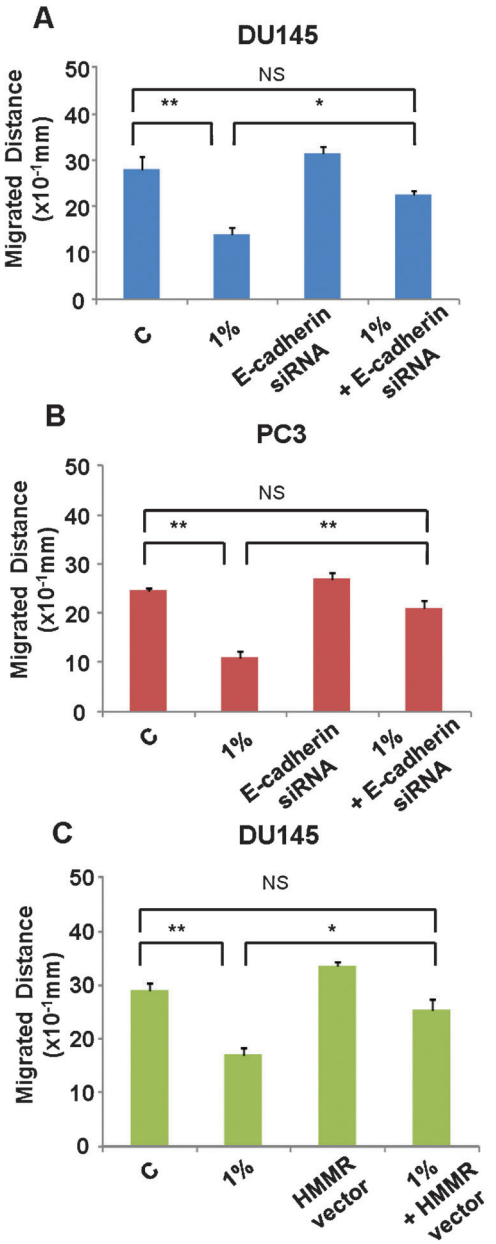


**Fig. 8 Effect of pomegranate juice on cell migration is mediated through E-cadherin and HMMR.** DU145 (A) and PC3 (B) prostate cancer cells were transfected with 40 nM E-cadherin siRNA and 24 h later a scratch made and treated with 1% PJ for 36 h and the distance migrated by the cells from the wounded edge to the leading edge was measured at the indicated time points. (C) DU145 cancer cells were transfected with 8 μg $ml^{-1}$ of pcDNA3.1 HMMR vector. Controls represent no PJ. Bars represent standard error of the mean. $^{**}p < 0.01$; $^{*}p < 0.05$. Experiment was performed two times.

expression of E-cadherin by inhibiting its transcriptional repression ZEB1 and ZEB through up-regulating miRNA-200 family. This suggestion is consistent with our mRNA microarray result that the expression level of E-cadherin is indeed increased by PJ. miRNA-126 was reported to reduce prostate cancer cell invasiveness by inhibiting prostein.[32] Prostein is a novel prostate-specific protein but its function is still largely unknown. miRNA-21 was previously identified

**List of selected miRNA changes with POMEGRANATE JUICE treatment**

| miRNA | Fold-change vs. Control | Predicted Target |
|---|---|---|
| **miR-335** | **↑ 411** | COL1A1, TNC, SOX4 |
| **miR-205** | **↑ 50** | CHN1, PRKCE |
| **miR-200 family** | **↑ 3-20** | ZEB1, ZEB2 |
| **miR-126** | **↑ 11** | SLC45A3 (Prostein) |
| **miR-21** | **↓ 12** | MARCKS, PDCD4, TPM1 |
| **miR-373** | **↓ 10** | CD44 |

**Fig. 9 Effects of pomegranate juice on the levels of metastasis-related miRNAs.** RNA from DU145 treated with 5% PJ for 12 h was submitted to microRNA array analysis. Relative miRNA levels are shown as fold change compared with untreated control. The predicted targets not mentioned in Fig. 9 are: tenascin C (TNC); SRY-related HMG-box (SOX-4); E-cadherin transcriptional repressor zinc finger E-box binding homeobox 1 (ZEB1) and zinc finger E-box binding homeobox 2 (ZEB2), resulting in up-regulation of E-cadherin; tropomyosin (TPM1); transmembrane adhesion glycoprotein cluster of differentiation 44 (CD44).

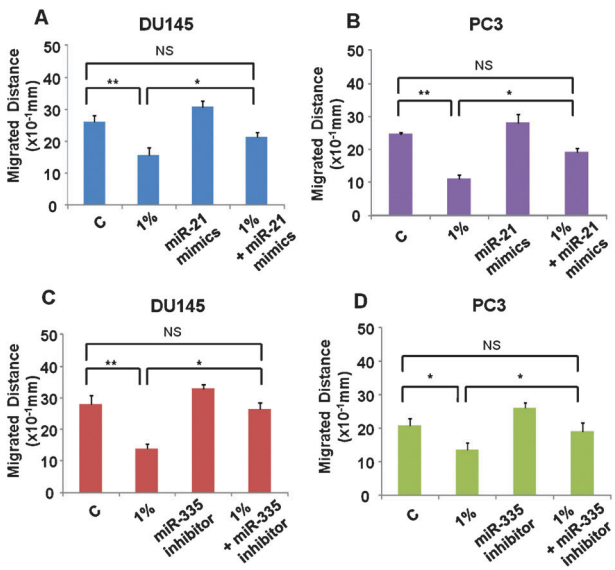


**Fig. 10 Effect of pomegranate juice on cell migration is mediated through miRNA-21 and miR-335.** DU145 (A) and PC3 (B) cells were transfected with 60 nM miR-21 mimics and 24 h later treated with 1% PJ for 36 h and the distance migrated by the cells from the wounded edge to the leading edge was measured at the indicated time points. DU145 (C) and PC3 (D) cells were transfected with 60 nM miR-335 inhibitor. Controls represent no PJ. Bars represent standard error of the mean. $^{**}p < 0.01$; $^{*}p < 0.05$. Experiment was performed two times

as a pro-invasive miRNA by inhibiting tropomyosin 1 (TMP1), programmed cell death 4 (Pdcd4) protein and MARCKS.[33] TPM1 is an actin-binding protein and its overexpression suppresses cell invasion.[34] Pdcd4 is a tumor

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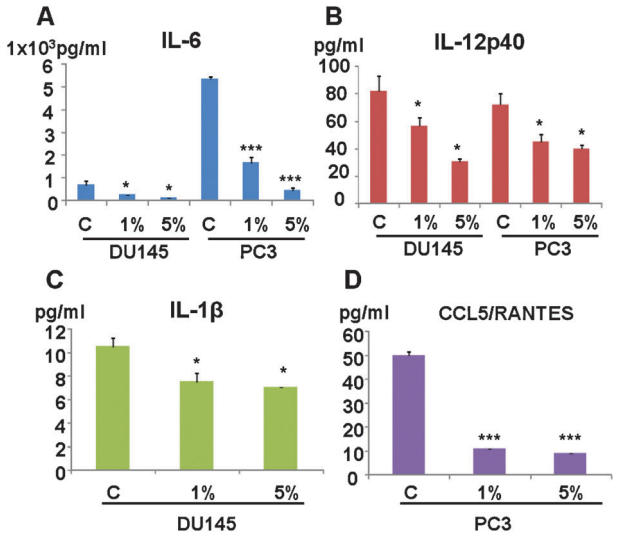


**Fig. 11 Effects of pomegranate juice on the levels of selected pro-inflammatory cytokines and chemokines.** Media collected from DU145 and PC3 cancer cells treated with 1% or 5% PJ for 18 h were analyzed using Luminex Multiplex Array assays. Both DU145 and PC3 significantly decreased IL-6 (A) and IL-12p40 (B) whereas only DU 145 inhibited production of IL-1β (C) and only PC3 inhibited production of RANTES (D). ***$p < 0.001$; *$p < 0.05$. Experiment was performed two times.

suppressor protein that is capable of interacting with eukaryotic initiation factor 4A (EIF4A) to inhibit protein synthesis.[35] Our mRNA microarray results show that the mRNA level of Pdcd4 and MARCKS are significantly down-regulated, which is consistent with inhibition by miRNA-21. Therefore, PJ might up-regulate TMP1, Pdcd4 and MARCKS through down-regulating miRNA-21. miRNA-373 was previously identified as a pro-invasive microRNA by targeting CD44, a transmembrane glycoprotein involved in cell adhesion and cell-stromal interactions.[36] CD44 also functions as a hyaluoronan receptor in addition to HMMR.[37] Therefore, the finding that PJ down-regulates miRNA-373 and HMMR suggests that the anti-metastatic effect of PJ may be partially due to inhibiting the hyaluoronan signaling pathway. In summary, PJ significantly up-regulates miRNA-335, miRNA-205 and the miRNA-200 family, all of which have been identified as metastasis suppressor miRNAs and significantly down regulates miRNA-21 and miRNA-373 that have been previously identified as pro-invasive miRNAs.

The molecular mechanisms of the anti-invasive effect of PJ are largely unknown. In addition to the genes and miRNAs we describe here that have the potential to mediate the PJ effects on adhesion and migration, we show that the PJ inhibition of prostate cancer cell migration occurs significantly through increasing E-cadherin and decreasing HMMR levels. We find that cells transfected with E-cadherin siRNA or with a vector that overexpresseses HMMR are no longer able to respond to PJ inhibition of migration. Moreover, we show that miR-21 mimics and a miR-335 inhibitor also partially reverse the inhibitory effect of PJ on cell migration.

***The cytokine array results*** show that pro-inflammatory cytokines/chemokines known to promote tumor growth and cancer progression are inhibited by PJ treatment. Among the 14 pro-inflammatory cytokines/chemokines examined with Luminex Multiplex Array assays, the secreted levels of IL-6, IL-12p40, IL-1β and RANTES are significantly reduced by PJ treatment. Elevated levels of IL-6 have been found in the tissues and serum of prostate cancer patients[38] and tumor growth was inhibited by anti-IL-6-antibody treatment.[39] Moreover, IL-6 is one of the cytokines that regulates angiogenesis and proliferation in prostate cancer through regulation of VEGF.[40] Heterodimeric pro-inflammatory cytokine IL-12 is composed of two subunits. The IL-12p40 subunit can act as a chemoattractant for macrophages and promotes migration of dendritic cells.[41] It has also been shown that colon cancer metastasis is inhibited by anti-IL-12p40-antibody treatment.[42] IL-1 is a pleiotropic cytokine that primarily affects inflammatory and immune responses, and also has important influence on diseases and malignancies. It has been reported that secreted IL-1β present in the microenvironment of the tumor cells stimulates inflammation that promotes invasion of fibrosarcoma cells.[43] RANTES (CCL5) is a potent chemotactic factor for T cells, monocytes and dendritic cells. Expression of RANTES and its receptor, CCR5, has been shown to correlate with prostate cancer progression. In addition, interaction of RANTES with CCR5 on the surface of cancer cells stimulates their invasive capabilities.[44] Our findings suggest that the observed anti-metastatic effects of PJ on prostate cancer cells are in part mediated through reducing the production of cancer-related pro-inflammatory cytokines and chemokines.

## Conclusion and future prospects

To date, there is no cure for prostate cancer when recurrence occurs after surgery and/or radiation. In particular, when it recurs after hormone ablation therapy there are no other effective treatments for deterrence of cancer progression. Here we have provided an overview of the myriad ways PJ appears to combat prostate cancer and perhaps other cancers, putting into perspective the job ahead. However, we know that there is a light at the end of the tunnel because the UCLA clinical trial has shown that at minimum PJ ameliorates prostate cancer progression and that there is a very high likelihood that it has a significant effect on metastasis in this disease. The data shown in this paper support and enhances that view. Because PJ interferes with multiple biological processes of cancer cells, stimulation of cell death as shown by ourselves and others, as well as increase in cell adhesion, decrease in cell migration and suppression of pro-inflammatory cytokines and chemokies shown here, PJ holds the promise of preventing or at least slowing down metastasis of prostate cancer. Discovery of the mechanisms by which enhanced adhesion, reduced migration and decrease of pro-inflammatory molecules are accomplished can potentially lead to more sophisticated and effective treatments of the disease when the component(s) of the juice responsible for these effects are identified. Pursuit of many of these aspects *in vivo* will dominate the work in our laboratory for the foreseeable future.

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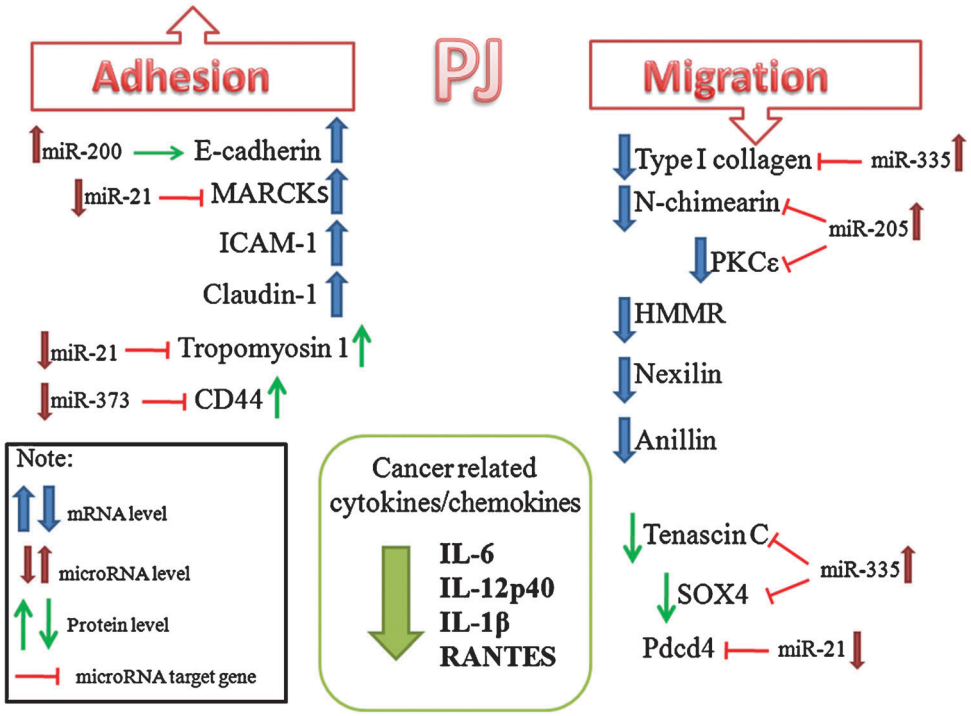


**Fig. 12 Schematic summary of the effects of pomegranate juice on hormone-independent prostate cancer cells.** E-cadherin is a calcium-dependent cell–cell adhesion glycoprotein and one of the important components in adherens junctions. Myristoylated alanine-rich protein kinase C substrate (MARCKS) is localized in the plasma membrane and is an actin filament cross-linking protein. Intercellular adhesion molecule-1 (ICAM-1) is an endothelial transmembrane protein known for its importance in cell adhesion and in stabilizing cell–cell interactions. Claudin-1, a transmembrane protein, is an important component of tight junctions. Type I collagen is an important component of ECM with both structural and signaling functions that mediate cell migration and survival. N-chimearin is known as a Rho GTPase-activating protein (GAP). Studies showed that N-chimearin cooperated with Rac1 in inducing changes in cytoskeletal morphology. Protein kinase Cε (PKCε) is a member of the protein kinase C family. Inhibition of PKCε reduces the invasiveness of prostate cancer cells. Hyaluronan-mediated motility receptor (HMMR) or CD168 functions as hyaluronan receptor. Anillin and nexilin are actin-binding proteins involved in regulation of cytoskeleton structure. Tenascin C is an extracellular molecule known to promote cell migration. Programmed cell death 4 (Pdcd4) has been identified as a tumor suppressor with the capability to suppress cancer cell invasion through JNK signaling pathway. miR-335 represses the expression of type I collagen and tenascin C. miR-205 represses the expression of N-chimearin and PKCε. N-chimearin is a GTPase-activating protein that when down-regulated results in loss of filopodia and reduction of migration. The miR-200 family represses E-cadherin transcriptional repressor ZEB1 and ZEB2, resulting in up-regulation of E-cadherin. miR-21 represses expression of MARCKS, tropomyosin 1 (TPM1), and Pdcd4. MARCKS and TPM1 are actin-filament binding proteins that are involved in the regulation of cell adhesion and cancer cell invasion, respectively. miR-373 represses transmembrane adhesion glycoprotein CD44 expression, a glycoprotein that mediates cell–cell and cell-stromal interactions, binds HA and several other matrix molecules, and controls cell shape through the cytoskeleton. PJ significantly up-regulates miR-335, miR-205, miR-200 family and miR-126 previously identified as metastasis suppressor microRNAs and significantly down regulated miR-21 and miR-373 previously identified as pro-invasive microRNAs.

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