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Gene Expression Changes in Pomegranate Peel Extract-Treated Triple-Negative Breast Cancer Cells

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Abstract

Background: Triple-negative breast cancer (TNBC) is treated with highly aggressive non-targeted chemotherapies. Safer and more effective therapeutic approaches than those currently in use are needed. Natural pomegranate peel extract (PPE) has recently been found to inhibit breast cancer progression; however, its mechanisms of action remain unclear. We hypothesized that transcriptional changes in the genes encoding the adherence proteins of intercellular adhesion molecule-1 (ICAM-1) and vascular endothelial growth factor (VEGF), may explain, at least in part, the anti-metastatic properties of PPE. Recently, the tumor microenvironment has been recognized as a key contributor to cancer progression. We speculated that PPE acts by modulating matrix glycoproteins including MMP9 and fibronectin. Moreover, we hypothesized that VEGF, which is required for tumor development, may contribute to the antimetastatic effects of PPE.

Methods: To address these possibilities, MDA-MB-231 cells were treated with different doses of PPE at different time points. Apoptosis was detected by flow cytometry using annexin V and propidium iodide. Cell migration was detected with a transwell assay. Gene expression changes were analyzed by real-time PCR.

Results: Exposure to PPE resulted in TNBC cell death and markedly inhibited PPE-resistant cell migration. Moreover, PPE up-regulated the expression of ICAM-1, a protein essential for cell adhesion, and down-regulated the expression of MMP9, fibronectin, and VEGF, the products of which contribute to cancer cell migration.

Conclusions: Transcriptional changes in ICAM-1, MMP9, fibronectin, and VEGF may contribute to PPE-mediated antimetastatic effects in TNBC.

Keywords: Gene Expression, Metastasis, Natural Product, Pomegranate Peel Extract (PPE), Triple Negative Breast Cancer.

Introduction

Breast cancer is the most commonly-diagnosed cancer and leading cause of cancer death among women worldwide (1). Breast cancer is a heterogeneous disease and one characteristic routinely used for its classification is its cell receptor status. The estrogen receptor (ER) is overexpressed in 60–70% of human breast cancers, and about 65% of ER-positive breast cancers are also progesterone receptor (PR) -positive. Human epidermal growth factor receptor 2 (HER2) is overexpressed in 15-20% of human breast cancers. However, triple-negative breast cancer (TNBC) lacks ER, PR, and HER2 biomarkers and constitutes 10–15% of all breast cancers (2). Those cancers that express ER, PR, or HER2 are responsive to targeted therapies directed at these receptors; however, because no targeted agents have been yet explored for TNBC, this subtype is often highly metastatic and patients have poor clinical outcomes (3). Although conventional drug therapy has been developed, until now, all drug options are toxic and none reduces the risk of disease progression for TNBC patients. The limited efficacy of current therapies against TNBC tumors has led to searches

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for novel combinatorial treatments. Recently, efforts to identify natural remedies to fight cancer have increased (4, 5). In a search for an agent that decreases progression of TNBCs, we evaluated pomegranate (*Punica granatum),* a medicinal plant containing bioactive phytochemicals that has been used to treat a variety of diseases including cancer (6). Pomegranate fruit can be divided into several anatomic compartments including seeds, juice, and peel, all of which have pharmacological activities. Modaeinama et al. showed that the peel extract of pomegranates native to Iran has significantly high phenolic and flavonoid contents. Moreover, 2,2-diphenyl-1-picrylhydrazyl (DPPH) IC50 analysis indicated high antioxidant activity of PPE (7). Several studies have shown that PPE has anti-proliferative and pro-apoptotic effects; however, little data is available regarding its antimetastatic effects (7, 8). Moreover, little is known about the molecular mechanisms affected by PPE that inhibit cancer progression, and in particular, those processes that lead to metastasis. It has recently been demonstrated that expression changes of cell adhesion molecules (CAMs) alter metastatic potential of cells. Accordingly, we hypothesized that transcriptional changes of molecules that affect cell adhesion, such as intercellular adhesion molecule 1 (ICAM-1) and vascular cell adhesion molecule 1 (VCAM-1) may explain, at least in part, the anti-metastatic properties of PPE. Another important element of mammary tumor progression to malignancy is the tumor microenvironment, which is created by the tumor and dominated by tumor-induced interactions (9). In this regard, we proposed that PPE may affect expression of matrix metalloproteinase 9 (MMP9) and fibronectin as key components of the tumor microenvironment. Moreover, vascular endothelial growth factor (VEGF) secretion is a prerequisite for tumor development. Although most of the effects of VEGF have been shown to be attributable to the stimulation of endothelial cells and angiogenesis, evidence also indicates that the VEGF-VEGFR1 signaling pathway is crucial for tumor metastasis (10).

To test these possibilities, MDA-MB-231 cancer cells as a TNBC model were treated with various concentrations of PPE at different time points. Thereafter, apoptosis, cell migration, and transcription of genes encoding products involved in cell adhesion and migration were analyzed. The aim of this study was to characterize the inhibitory effects of PPE on cancer progression and metastasis in MDA-MB-231 cells. Identification of potential PPE targets may lead to effective use of pomegranate for breast cancer treatment.

Materials and methods

PPE preparation

Pomegranate skins were isolated, dried, ground into powder, and extracted with methanol. The extracts were concentrated under vacuum at 40°C and stored at -20°C.

Cell culture

The human metastatic breast cancer cell line, MDA-MB-231, was obtained from the National Cell Bank of Iran (Pasteur Institute of Iran, Tehran, Iran). Cells were cultured at 37 °C in a 5% CO2 humidified atmosphere in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal calf serum (FBS) and 1% penicillin-streptomycin at 10,000 units/ml.

Apoptosis assay

The cells were seeded in 6-well microplates and allowed to proliferate until the confluency reached 70–80%. Cells were incubated with 12.5, 25, 50, 100, 250, 500, and 1000 µg/ml of PPE for 24h, 48h, and 72h. Then the media were removed and cells were suspended in 500 µL of binding buffer. The cells were then incubated with annexin V-FITC and propidium iodide (PI) in the dark for 15 min at room temperature. Early and late apoptotic and necrotic cell populations were determined by constructing a dot-plot with the aid of fluorescence-activated cell sorting.

Migration assay

The transwell migration assay was performed as described before (11). Briefly, MDA-MB-231 cells were plated on the upper side of a polycarbonate membrane of a transwell chamber in medium without serum. The cells were washed twice with PBS, incubated with 12.5, 25, 50, 100, 250, 500, and 1000 µg/ml of PPE for 24 h, fixed and then stained with 4',6-diamidino-2-phenylindole (DAPI). Four random fields of cells migrating through the membrane were counted on an Olympus IX71 fluorescence microscope.

RNA extraction and real-time PCR

Total RNA was isolated from the PPE-treated MDA-MB-231 cells using TriPure Isolation Reagent (Roche, Germany) following the manufacturer's instructions. To remove genomic DNA contamination, the extracted RNAs were treated with DNase I. To synthesize cDNA, 1 µg of RNA was mixed with 1 µl of oligo (dT) (0.5 µg/µl) and incubated at 65˚C for 5 min. After cooling on ice, each sample was mixed with 4 µl of 5X buffer, 2 µl of 10 mM dNTPs, 0.5 µl of RiboLock, and 1 µl of M-MLV-RTase, and incubated at 42 ˚C for 60 min, followed by 70˚C for 10 min. The synthesized cDNAs were diluted in distilled water (1:3) and 2 µl of the diluted cDNA samples were mixed with real-time PCR reaction mixture containing 10 µl of 2X SYBR Green PCR Master Mix (Parstous, Iran) and 1 µl of specific primer pairs in final 20 µl volumes. Samples were incubated at 95 ˚C for 10 min, then amplified by 40 cycles of 95 ˚C for 15 sec, 60 ˚C for 30 sec, and 72 ˚C for 30 sec (12). Primers used in this study are shown in Table 1. The housekeeping gene, glucuronidase beta (GusB), was used to normalize target gene expression. The comparative expression level of each target gene was calculated by $2^{-\Delta\Delta CT}$. Melting curves were used to determine non-specific amplification.

Table 1. Real-time PCR primer sequences.

Primers	sequence	Product size
ICAM-1	F: AGGCCACCCCAGAGGACAAC R: CCCATTATGACTGCGGCTGCTA	406 bp
VCAM-1	F: CGTCTTGGTCAGCCCTTCCT R: ACATTCATATACTCCCGCATCCTTC	460 bp
MMP9	F: GCACGACGTCTTCCAGTACC R: CAGGATGTCATAGGTCACGTAGC	124 bp
Fibronectin	F: TCCTTGCTGGTATCATGGCAG R: AGACCCAGGCTTCTCATACTTGA	74 bp
VEGF	F: CCTTGCTGCTCTACCTCCAC R: ATCTGCATGGTGATGTTGGA	280 bp
GusB	F: CTC ATT TGG AAT TTT GCC GAT T R: CCG AGTGAA GAT CCC CTT TTT A	81 bp

Statistical analysis

The data were analyzed and visualized using GraphPad Prism version 6.0. Differences between the groups were assessed by one-way ANOVA followed by Dunnett's test. Data are presented as means ± standard deviations (SDs).

Results

The effects of PPE on apoptosis of MDA-MB-231 cells

To investigate whether PPE affects TNBC cell apoptosis, cells were dual-labeled with Annexin V-FITC and PI. After PPE treatment for 24, 48, and 72 h, the percentages of the early and late apoptotic and necrotic cells were increased (Fig. 1).

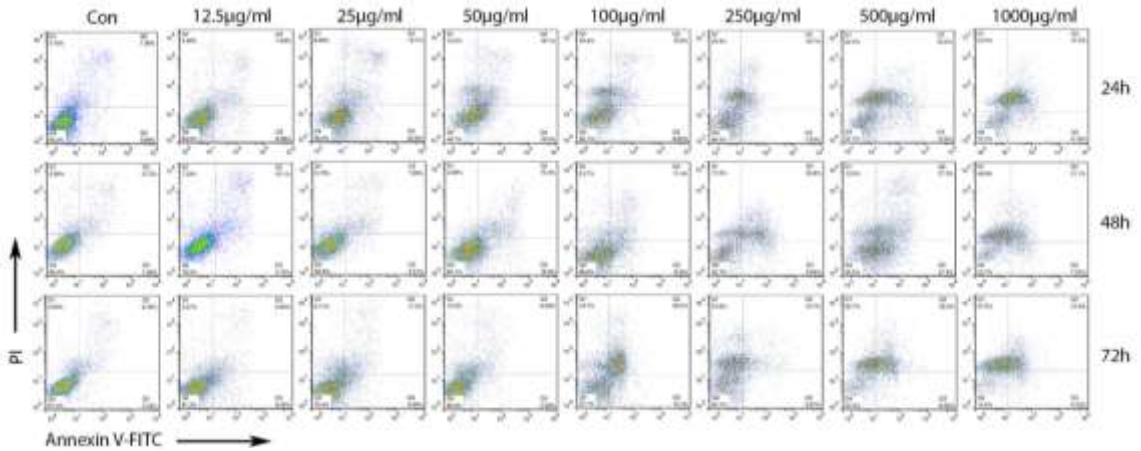

Fig. 1. Flow cytometry with Annexin V and propidium iodide staining. Cells were treated with PPE at the indicated doses for 24, 48, and 72 h and apoptosis was assessed by flow cytometry. The four quadrants represent living cells (Annexin V-PI-), early apoptotic (Annexin V+PI-), late apoptosis (Annexin+PI+) or necrotic (Annexin V-PI+) stages. Values shown are percentages of each quadrant.

Cells treated with 500 or 1000 µg/ml of PPE for 24, 48, and 72 h were significantly more apoptotic than controls (data not shown).

The effects of PPE on migration motility in MDA-MB-231 cells

Cell migration was evaluated using a two-chamber transwell system. Cells were cultured with the various doses of PPE for 24 h and cells that had traversed the membrane to the bottom side were fixed and stained. The results showed that PPE inhibited cell migration in a dose-dependent manner (Fig. 2).

PPE altered metastasis-associated gene expression

Exposure of MDA-MB-231 cells to PPE for 48 and 72 h increased ICAM-1 and decreased MMP9, fibronectin, and VEGF mRNA expression in a dose-dependent manner. The 72-hour result is shown in Figure 3 (Fig. 3). PPE had no effect onVCAM-1 expression (data not shown).

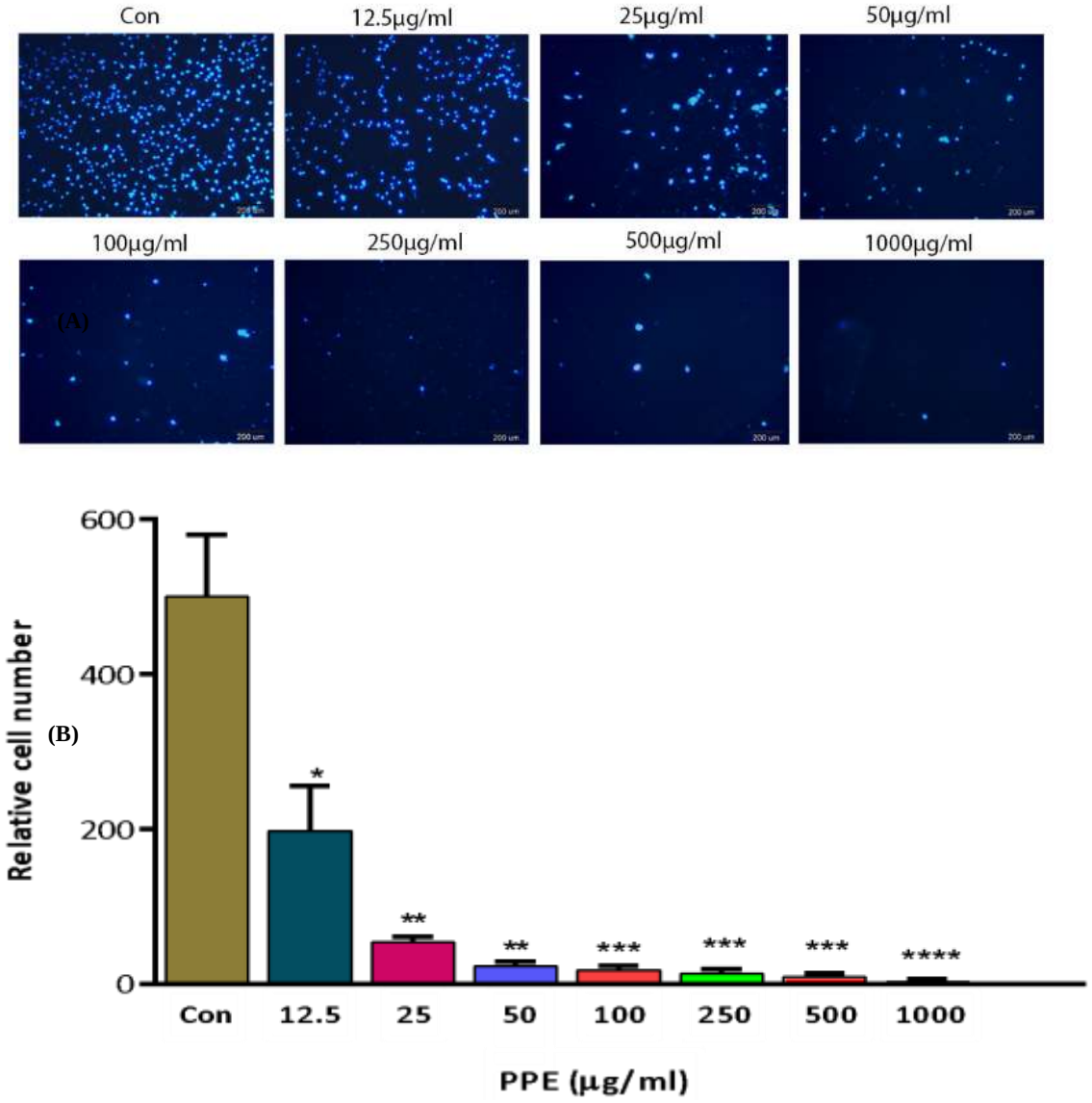

Fig. 2. PPE inhibits MDA-MB-231 cell migration. (A) Cells on the transwell filter that migrated to the lower surface of the filter were fixed and then stained with DAPI and photographed on a light microscope. The bars represent 200 µm. (B) The results are an average of the number of migrated cells on the underside of the filter. Significance was set at *P < 0.05, **P < 0.01, ***P < 0.001, ****P < 0.0001.

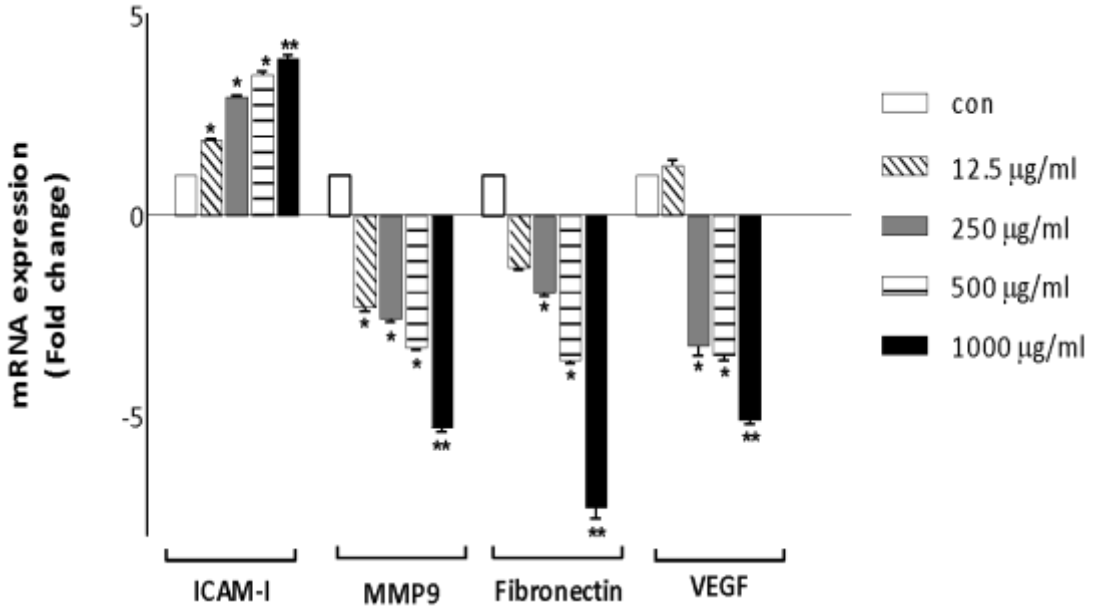

Fig. 3. PPE modulates gene expression in MDA-MB-231 cells. Total RNA from PPE-treated and untreated MDA-MB-231 cells was extracted after 72 h of incubation, cDNA was synthesized, and real-time PCR was performed. ICAM-1 was upregulated while MMP9, fibronectin, and VEGF were downregulated by PPE in a dose-dependent manner. The data represent the means ± SDs (*P < 0.05, **P < 0.01).

Discussion

This study identified genes that might serve as biomarkers involved in the antimetastatic property of PPE on TNBC cells. We showed that PPE inhibits TNBC cell migration and stimulates the expression of genes involved in cell adhesion while reducing the expression of genes involved in cell invasion and migration.

Recently, it has been found that expression changes of several cell adhesion molecules alter the metastatic potential of cells. Among these is ICAM-1, a transmembrane glycoprotein in the immunoglobulin superfamily. In a clinicopathologic analysis of colorectal cancer, a correlation was found between ICAM-1 expression and patients' prognoses. Patients with ICAM-1-negative tumors had significantly poorer prognoses than those with ICAM-1-positive tumors (13). Injection of colon cancer cells into ICAM-1-deficient mice promoted tumor progression and metastasis, whereas no change in tumor cell proliferation was reported (14). These findings indicate that ICAM-1 may have an important role in initiation and progression of metastasis. Wang et al. showed that treatment of prostate cancer cells with pomegranate juice upregulated genes involved in cell adhesion such as ICAM-1 (15). In our study, the finding that PPE upregulates ICAM-1 suggests that the anti-metastatic effect of PPE may be partially due to increased ICAM-1 expression.

Vascular cell adhesion molecule 1, expressed on endothelial cells under inflammatory conditions, mediates leukocyte rolling, adhesion, and transmigration from the inner surface of blood vessels into tissue (16). Moreover, some cancer cell types express VLA-4, a ligand for VCAM-1. Interaction of VLA-4-positive tumor cells in the circulation with VCAM-1 on the activated endothelium enhances their transmigration into the subendothelium to make a new tumor site (17). In addition to endothelial expression of VCAM-1 and its role in metastasis, some cancer cell types also express VCAM-1. These cancer cells, by attracting VLA-4-positive monocytes and macrophages, are protected from immune surveillance (18). Additionally, binding of macrophages to VCAM-1 enhances survival signals in breast cancer cells that invade the lungs (19). Significant reductions in serum (soluble) VCAM-1 was reported after pomegranate juice intake (20). We found no decrease in VCAM-1 expression in TNBC cells. Further investigation should be performed to determine the mechanism by which pomegranate alters VCAM-1 expression.

The extracellular matrix (ECM) is a protein-rich environment that serves many functions including

tissue support, regulation of cell-cell adhesion and communication, and support for cell chemotaxis and migration (21). A large body of evidence demonstrates that ECM proteins are aberrantly expressed in human cancers and actively contribute to tumor progression. In particular, the matrix metalloproteinases (MMPs), as major ECM components, have received increasing interest due to their participation in multiple stages of tumor progression. MMPs degrade and remodel the ECM, allowing tumor cell migration (22, 23). MMP9, an MMP family member, was introduced as a poor prognosis signature for breast cancer patients (24). MMP9 knockdown and gene silencing experiments showed reduced invasiveness and tumor growth in TNBC cells (24). Our data, in which PPE decreased MMP9 expression, suggests PPE as a drug that targets MMP9.

Fibronectin, another structural ECM component, is over-expressed in several cancers and has been shown to promote tumor growth and invasion (25). Overexpression of SOX2, through upregulation of fibronectin, increased cell migration and invasion in ovarian cancer cells (26). Wei et al. showed that nicotine increased colon cancer cells migration through upregulation of fibronectin. It was also shown that COX-2 signaling was involved in this induction (27). Knowles et al. found that fibronectin and integrin αvβ3 induced lung metastasis through upregulation of the transcription factor Slug, which mediates epithelial-mesenchymal transition (28). Balanis et al. showed that adhesion of MDA-MB-231 breast cancer cells to fibronectin promotes cancer progression through a fibronectin-dependent STAT3 signaling pathway (29). Here, decreased expression of fibronectin may indicate its effect on metastasis inhibition after PPE exposure in TNBC cells.

Targeting VEGF signaling is an attractive approach to prevent tumor metastasis because cancer cells require angiogenesis to metastasize. Angiogenesis provides nutrients to tumor cells and provides them the opportunity to enter the circulation to form distant metastases. Tumor angiogenesis is elicited and regulated by several factors including VEGF-A, which is secreted by tumor cells and plays an important role in angiogenesis (30). Evidence from preclinical and clinical studies indicates that VEGF is predominantly associated with the formation of metastatic foci and poor prognosis (30, 31).VEGF binding to its receptor induces receptor dimerization and autophosphorylation, leading to activation of some signaling pathways including MAPK and ERK, which are involved in cell proliferation, survival, and migration (31). Decreased VEGF expression after treatment with PPE treatment suggests it may contribute to PPE's antimetastatic effects.

The outlook for pomegranate as an antimetastatic drug can be improved by unraveling the mechanisms that mediate its antimigratory properties and identifying its novel targets. To our knowledge, this is the first report on the transcriptional changes of some key genes involved in metastasis after exposure of TNBC cells to PPE, and these results may at least partly explain the underlying mechanisms of this effect.

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References

1. Siegel RL, Miller KD, Jemal A. Cancer statistics, 2016. CA: a cancer journal for clinicians. 2016;66(1):7-30.

2. Tang P, Tse GM. Immunohistochemical Surrogates for Molecular Classification of Breast Carcinoma: A 2015 Update. Arch Pathol Lab Med. 2016 Aug;140(8):806-14.

3. Kennecke H, Yerushalmi R, Woods R, Cheang MCU, Voduc D, Speers CH, et al. Metastatic behavior of breast cancer subtypes. Journal of clinical oncology. 2010;28(20):3271-7.

4. Ahmadiankia N, Moghaddam HK, Mishan MA, Bahrami AR, Naderi-Meshkin H, Bidkhori HR, et al. Berberine suppresses migration of MCF-7 breast cancer cells through down-regulation of chemokine receptors. Iran J Basic Med Sci. 2016 Feb;19(2):125-31.

5. Mishan MA, Ahmadiankia N, Matin MM, Heirani-Tabasi A, Shahriyari M, Bidkhori HR, et al. Role of Berberine on molecular markers involved in migration of esophageal cancer cells. Cell Mol Biol (Noisy-le-grand). 2015 Dec 14;61(8):37-43.
6. Turrini E, Ferruzzi L, Fimognari C. Potential Effects of Pomegranate Polyphenols in Cancer Prevention and Therapy. Oxid Med Cell Longev. 2015; 2015:938475.
7. Modaeinama S, Abasi M, Abbasi MM, Jahanban-Esfahlan R. Anti Tumoral Properties of Punica Granatum (Pomegranate) Peel Extract on Different Human Cancer Cells. Asian Pac J Cancer Prev. 2015;16(14):5697-701.
8. Orgil O, Spector L, Holland D, Mahajna J, Amir R. The anti-proliferative and anti-androgenic activity of different pomegranate accessions. Journal of Functional Foods. 2016;26:517-28.
9. Whiteside TL. The tumor microenvironment and its role in promoting tumor growth. Oncogene. 2008 Oct 6;27(45):5904-12.
10. Liu W, Xu J, Wang M, Wang Q, Bi Y, Han M. Tumor-derived vascular endothelial growth factor (VEGF)-a facilitates tumor metastasis through the VEGF-VEGFR1 signaling pathway. Int J Oncol. 2011 Nov;39(5):1213-20.
11. Mirahmadi M, Ahmadiankia N, Naderi-Meshkin H, Heirani-Tabasi A, Bidkhori HR, Afsharian P, et al. Hypoxia and laser enhance expression of SDF-1 in muscles cells. Cell Mol Biol (Noisy-le-grand). 2016 Apr 30;62(5):31-7.
12. Mishan MA, Heirani-Tabasi A, Mokhberian N, Hassanzade M, Kalalian Moghaddam H, Bahrami AR, et al. Analysis of Chemokine Receptor Gene Expression in Esophageal Cancer Cells Compared with Breast Cancer with Insights into Metastasis. Iran J Public Health. 2015 Oct;44(10):1353-8.
13. Maeda K, Kang SM, Sawada T, Nishiguchi Y, Yashiro M, Ogawa Y, et al. Expression of intercellular adhesion molecule-1 and prognosis in colorectal cancer. Oncol Rep. 2002 May-Jun;9(3):511-4.
14. Yang M, Liu J, Piao C, Shao J, Du J. ICAM-1 suppresses tumor metastasis by inhibiting macrophage M2 polarization through blockade of efferocytosis. Cell Death Dis. 2015 Jun 11;6:e1780.
15. Wang L, Alcon A, Yuan H, Ho J, Li QJ, Martins-Green M. Cellular and molecular mechanisms of pomegranate juice-induced anti-metastatic effect on prostate cancer cells. Integr Biol (Camb). 2011 Jul; 3(7):742-54.
16. Wittchen ES. Endothelial signaling in paracellular and transcellular leukocyte transmigration. Front Biosci (Landmark Ed). 2009 Jan 1; 14:2522-45.
17. Schlesinger M, Bendas G. Vascular cell adhesion molecule-1 (VCAM-1) –an increasing insight into its role in tumorigenicity and metastasis. Int J Cancer. 2015 Jun 1;136(11):2504-14.
18. Lin KY, Lu D, Hung CF, Peng S, Huang L, Jie C, et al. Ectopic expression of vascular cell adhesion molecule-1 as a new mechanism for tumor immune evasion. Cancer Res. 2007 Feb 15;67(4):1832-41.
19. Chen Q, Zhang XH, Massague J. Macrophage binding to receptor VCAM-1 transmits survival signals in breast cancer cells that invade the lungs. Cancer Cell. 2011 Oct 18;20(4):538-49.
20. Asgary S, Keshvari M, Sahebkar A, Hashemi M, Rafieian-Kopaei M. Clinical investigation of the acute effects of pomegranate juice on blood pressure and endothelial function in hypertensive individuals. ARYA Atheroscler. 2013 Nov;9(6):326-31.
21. Jarvelainen H, Sainio A, Koulu M, Wight TN, Penttinen R. Extracellular matrix molecules: potential targets in pharmacotherapy. Pharmacol Rev. 2009 Jun;61(2):198-223.
22. Stamenkovic I. Extracellular matrix remodelling: the role of matrix metalloproteinases. J Pathol. 2003 Jul;200(4):448-64.
23. Kessenbrock K, Plaks V, Werb Z. Matrix metalloproteinases: regulators of the tumor microenvironment. Cell. 2010 Apr 2;141(1):52-67.
24. Merdad A, Karim S, Schulten HJ, Dallol A, Buhmeida A, Al-Thubaity F, et al. Expression of matrix metalloproteinases (MMPs) in primary human breast cancer: MMP-9 as a potential biomarker for cancer invasion and metastasis. Anticancer Res. 2014 Mar; 34(3):1355-66.

25. Wang JP, Hielscher A. Fibronectin: How Its Aberrant Expression in Tumors May Improve Therapeutic Targeting. J Cancer. 2017;8(4):674-82.
26. Lou X, Han X, Jin C, Tian W, Yu W, Ding D, et al. SOX2 targets fibronectin 1 to promote cell migration and invasion in ovarian cancer: new molecular leads for therapeutic intervention. Omics. 2013 Oct;17(10):510-8.
27. Wei PL, Kuo LJ, Huang MT, Ting WC, Ho YS, Wang W, et al. Nicotine enhances colon cancer cell migration by induction of fibronectin. Ann Surg Oncol. 2011 Jun;18(6):1782-90.
28. Knowles LM, Gurski LA, Engel C, Gnarra JR, Maranchie JK, Pilch J. Integrin alphavbeta3 and fibronectin upregulate Slug in cancer cells to promote clot invasion and metastasis. Cancer Res. 2013 Oct 15;73(20):6175-84.
29. Balanis N, Wendt MK, Schiemann BJ, Wang Z, Schiemann WP, Carlin CR. Epithelial to mesenchymal transition promotes breast cancer progression via a fibronectin-dependent STAT3 signaling pathway. J Biol Chem. 2013 Jun 21;288(25):17954-67.
30. Bendardaf R, El-Serafi A, Syrjänen K, Collan Y, Pyrhönen S. The effect of vascular endothelial growth factor-1 expression on survival of advanced colorectal cancer patients. Libyan Journal of Medicine. 2017;12(1):1290741.
31. Roberts E, Cossigny DA, Quan GM. The role of vascular endothelial growth factor in metastatic prostate cancer to the skeleton. Prostate Cancer. 2013;2013:418340.