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**The effect of pomegranate juice extract on the Hedgehog signaling pathway in pancreatic cancer**

Veronica Gomez, Talia Shackelford, Autumn Tocchi, Melissa Rowland-Goldsmith Ph.D.

**Abstract**

Pancreatic cancer is the fourth leading cause of cancer death in the United States. There have been several reports indicating that phytochemicals in fruits can reduce the risk of cancer due to the anti-oxidant and anti-inflammatory effects of the polyphenols. Our lab has shown that pomegranate juice extract (PJE) has anti-proliferative and pro-apoptotic effects in human pancreatic cancer cells. In the past, we have shown that cells adhere more strongly to the plate when treated with PJE. This observation prompted an investigation of how PJE regulates cell adhesion proteins. Previously, our lab investigated E-cadherin, a cell adhesion protein. Upon activation of the Hedgehog signaling pathway, Gl-1 has been shown to down-regulate E-cadherin. The purpose of this study was to determine if PJE up-regulates ezrin, another cell adhesion protein, by interfering with the Gl-1 transcription factor of the Hedgehog signaling cascade. Through the use of immunoblots, we evaluated Gl-1 and ezrin protein levels after PJE treatment in COLO-357 human pancreatic cancer cells. We showed that pancreatic cancer cells treated with PJE led to decreased expression of Gl-1 and up-regulation of ezrin. This data suggests that PJE can help restore pancreatic cancer cell adhesion by blocking an important signaling pathway, thus serving as a potential suppressor of invasion and metastasis.

**Keywords:** Pomegranate juice extract, pancreatic cancer**Introduction**

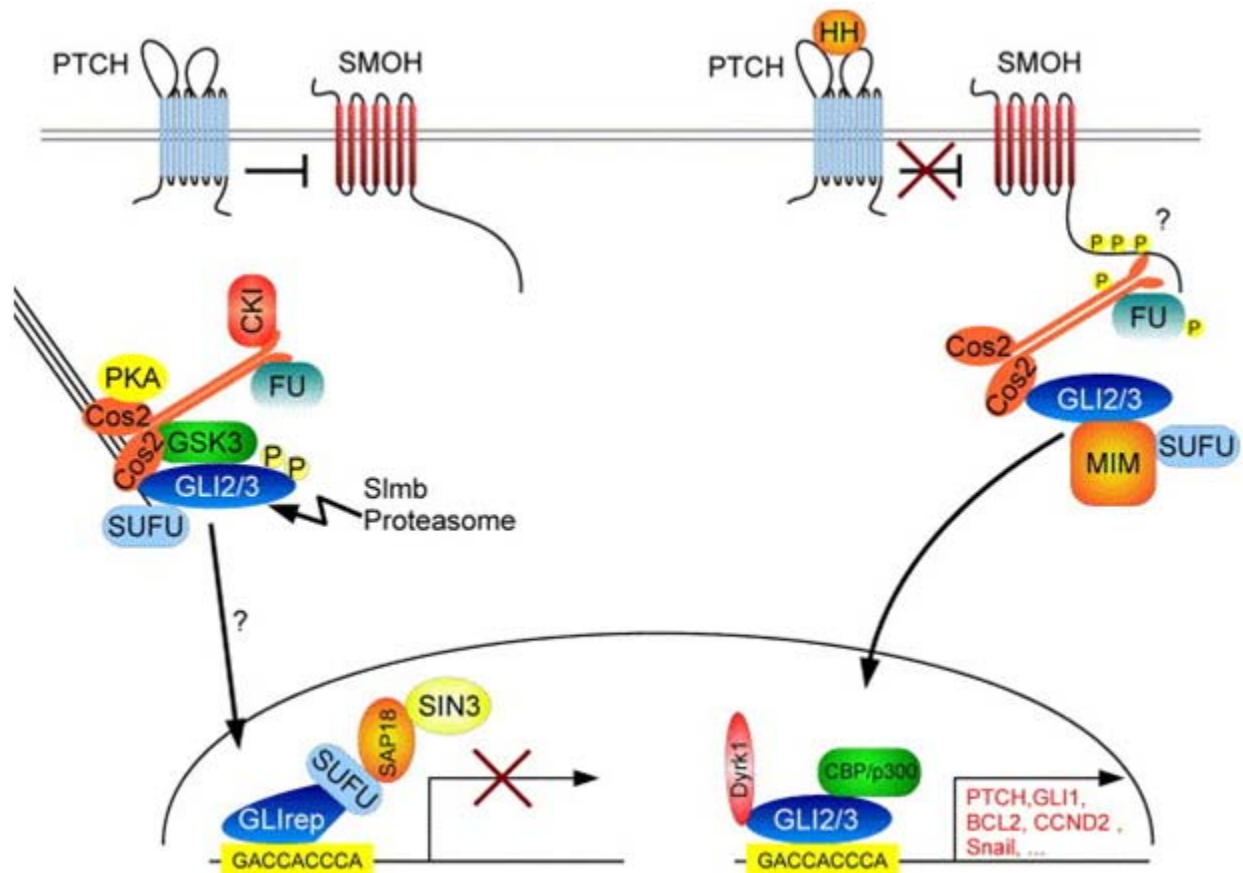
Pancreatic ductal adenocarcinoma is a devastating disease in which the overall five year survival rate is approximately 3-5% (1). Non-surgical treatment is generally ineffective due to the resistance of pancreatic cancer cells to chemotherapy and the tumor's ability to metastasize (1).

The demand for dietary alternatives has prompted explorative studies on phytochemicals, which have been shown to be effective in fighting other cancers (2-13). Phenolic acids, flavanoids, and polyphenols are subgroups of phytochemicals, which are found in pomegranate juice extract (PJE) (12). Their ability to act as antioxidants makes them valuable agents in cancer therapies. Other studies have provided evidence for pomegranate juice extract's anti-inflammatory, antioxidant, chemotherapeutic, chemo-preventive, anti-proliferative, and pro-apoptotic properties (2-12). Recently, the complete pancreatic cancer genome sequence has been determined in which 12 signaling pathways involved in promoting the disease were identified (14).

One of these pathways is called the Hedgehog (HH) signaling pathway which has been found to be defective in patients with pancreatic cancer (15-17). Under normal circumstances, as seen in Fig. 1, the Hedgehog pathway

V. Gomez, T. Shackelford, A. Tocchi, M. Rowland-Goldsmith

begins when the HH-protein binds to its receptor Patched (Ptc) (15). When HH is repressed the transmembrane protein Smoothened (Smo) is inhibited (15). When the HH protein binds to its receptor, Smo is activated and transduces a signal, thereby activating Gl-1 transcription factor. Gl-1 then translocates to the nucleus where it regulates gene transcription (15). In many cancers, this pathway is defective, causing an increase in HH protein and an over expression of the Gl-1 (15-17).



**Figure 1:** Proposed mechanism of the Hedgehog Signaling pathway showing activation through HH protein binding (15).

In order for tumors to become metastatic, they must exhibit reduced cell-cell adhesion, alteration of tumor-extracellular matrix interaction, and invasion of surrounding tissue (18). Low expression of E-cadherin correlates with increased pancreatic cancer proliferation and metastasis (19). Previous studies suggest that restoring these protein levels in pancreatic cancer cells promotes cell adhesion, leading to an increased rate in apoptosis (19). E-cadherin and ezrin are two cell adhesion proteins that are involved in pancreatic cancer (16,19-22). In pancreatic cancer, E-cadherin is down-regulated by Gl-1 (16). Our laboratory has previously shown that PJE up-regulates E-cadherin levels (data not shown). The present study was conducted to determine if pancreatic cancer cells treated with PJE up-regulates ezrin and whether it does so by interfering with the Gl-1 transcription factor.

**Methods**

Cell Culture: COLO-357 human pancreatic cancer cells (Dartmouth University) were grown in Dulbecco's Modified Eagle's (DME) complete media (CellGro) containing 10% fetal bovine serum (Irvine Scientific), 0.25 µg/ml fungazone (Omega Scientific), 100 µg/ml penicillin and 100 µg/ml streptomycin (Biowhitaker). Cells were

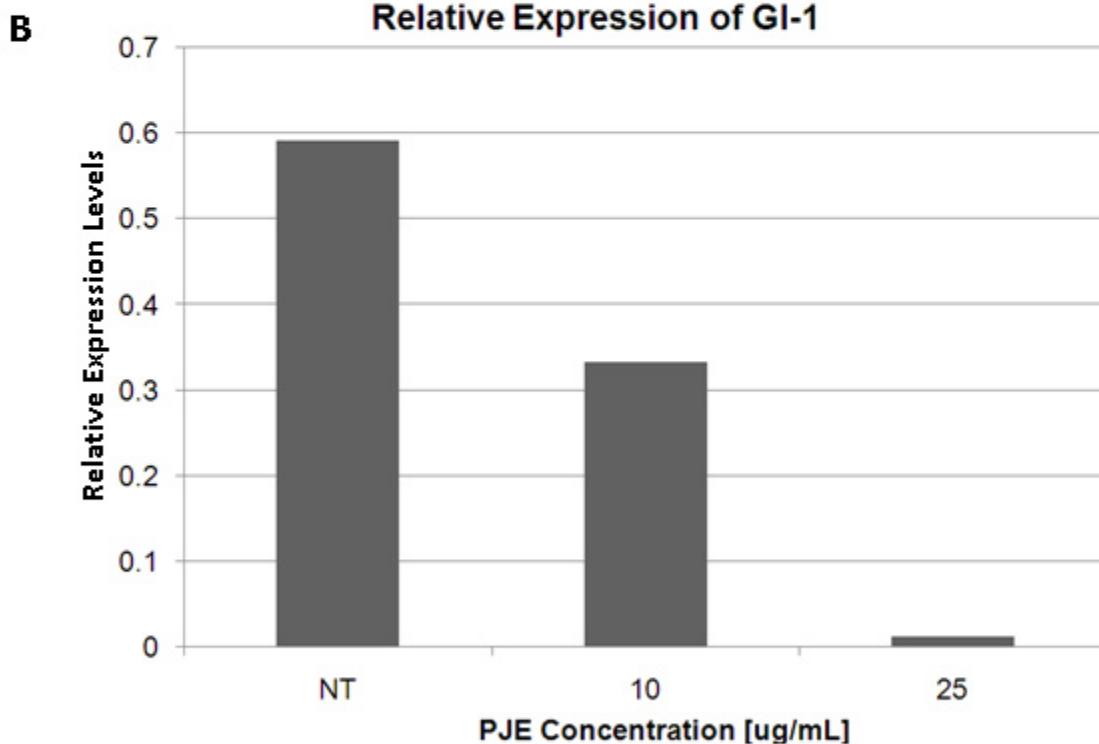
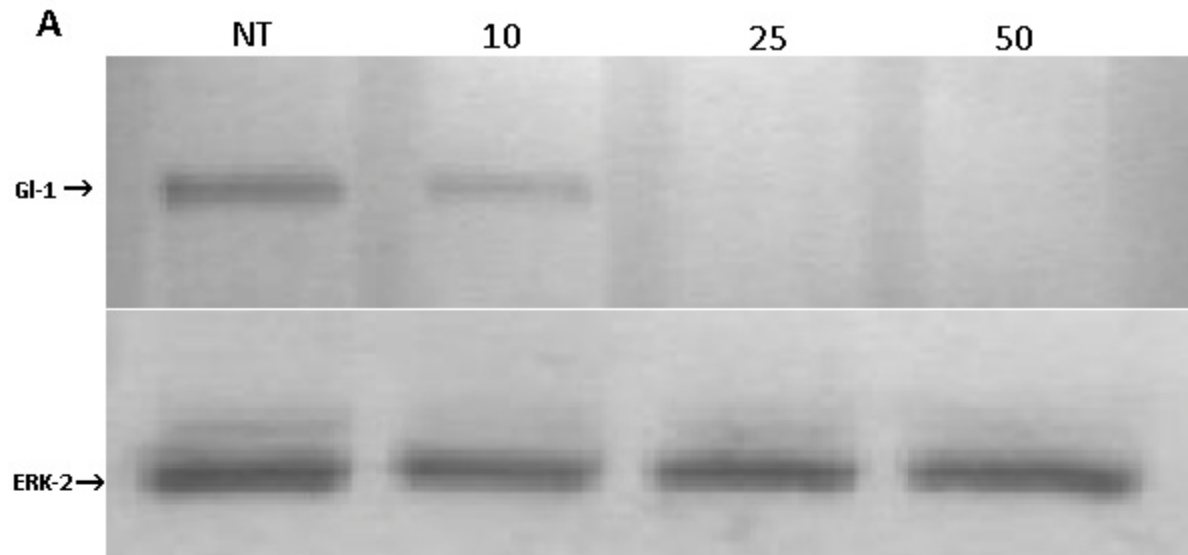
maintained in a humidified 5% CO<sub>2</sub> and 95% air atmosphere at 37 degrees C. Confluent cells were removed from the plate using trypsin-EDTA (CellGro).

Preparation of Cells for Experiments: Confluent cells were removed from the plate using trypsin-EDTA.  $8 \times 10^5$  cells per well were seeded into 6 well plates and grown overnight in DME complete media. Plates were then grown overnight in DME serum free media containing 0.25 µg/ml fungazone, 100 µg/ml penicillin, 100 µg/ml streptomycin, and 1X ITS (insulin, transferrin, selenium) (Biowhitaker) before treatment. Cells were then treated with various concentrations of POM Wonderful PJE (48 hours) in triplicates: no treatment (NT), 10ug/ml PJE, 25ug/ml PJE, and 50 ug/ml PJE.

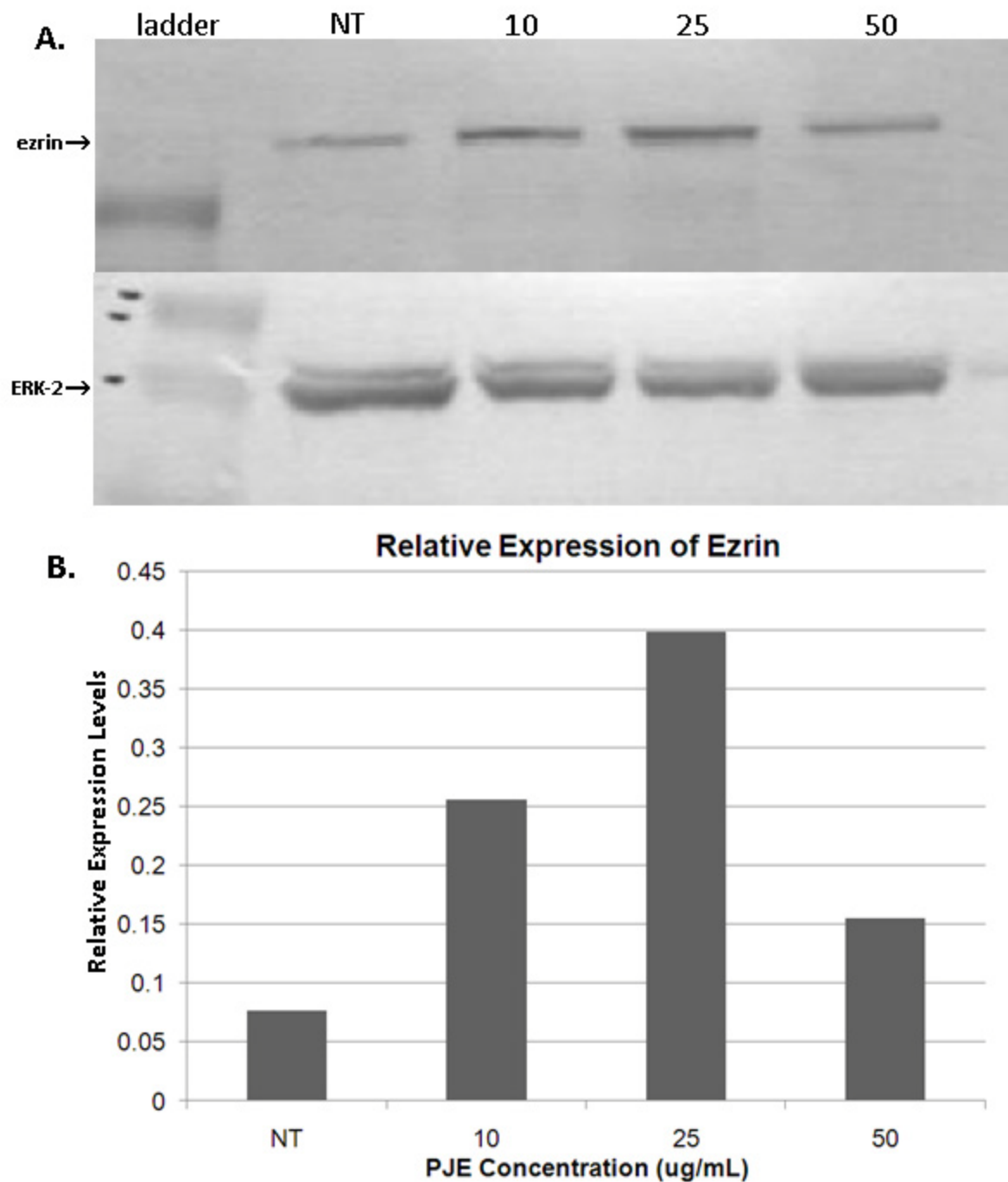
Immunoblotting: After incubation, protein lysates were collected and the protein concentrations were determined using a BCA assay (Thermo Scientific). The samples were run, along with a pre-stained BenchMark molecular weight marker (Invitrogen), on a 7.5% SDS-PAGE gel (BioRAD), electrotransferred to an Immobilon P membrane (Millipore), and blotted with a goat anti-rabbit ezrin (Santa Cruz) 1:500 dilution or goat anti-rabbit Gl-1 (Santa Cruz) 1:200 dilution. Goat anti-rabbit ERK2 (Santa Cruz) was used at a 1:1,330 dilution as a loading control.

Images: Images of immunoblots were taken using Fotodyne 60-0300 (Fotodyne Inc). Volumetric analysis was performed on images using the Total Lab Software (Nonlinear Dynamics Ltd).

Results



**Figure 2: Gl-1 protein expression was decreased by PJE treatment in pancreatic cancer cells.** 2a. Pancreatic cancer cell lysates (20ug) were prepared from cells alone (NT); 10 ug/mL PJE; 25 ug/mL PJE; and 50 ug/mL PJE for 48 hrs. They were then subjected to 7.5% SDS-PAGE, electrotransferred to a membrane, and blotted with the anti-Gl-1 antibody (1:200 dilution) or anti ERK-2 antibody (1:1,330 dilution) which was used as a loading control. The western blot was detected using the Western Breeze kit. 2b. Expression levels were determined using Total Lab analysis. Note: there was not a band for the 50 ug/ml PJE treatment so the relative expression could not be computed.



**Figure 2: Ezrin protein expression was increased by PJE treatment in pancreatic cancer cells.** 3a. Pancreatic cancer cell lysates (10ug) were prepared from cells alone (NT); 10 ug/mL PJE; 25 ug/mL PJE; and 50 ug/mL PJE for 48 hrs. They were then subjected to 7.5% SDS-PAGE, electrotransferred to a membrane, and blotted with the anti-ezrin antibody (1:500 dilution) or anti ERK-2 antibody (1:1,330 dilution) which was used as a loading control. The western blot was detected using the western breeze kit. 3b. Expression levels were determined using Total Lab analysis.

V. Gomez, T. Shackelford, A. Tocchi, M. Rowland-Goldsmith

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There was a dose dependent decrease in GI-1 when cells were treated with PJE (Figure 2). Ezrin protein levels had a dose dependent increase up to 25 µg/mL PJE treatment (Figure 3). There was an increase in the PJE treatment of 50 µg/mL, however, it was weaker compared to the 25 µg/mL PJE treatment (Figure 3).

## Discussion

We have shown in past research that the most prominent polyphenols in POM Wonderful PJE are Quinic acid, Ellagic acid, Punicalin, and Punicalagin (data not shown). Ellagic acid has pro-apoptotic effects in pancreatic cancer (23). Formerly, we found that cancer cells treated with PJE led to decreased cell growth. This data partially explains why the cancer cells became more adherent to the culture dish. Since Ellagic acid is the only commercially available polyphenol found in pomegranates, we replicated the above experiments with this single polyphenol and found that the cancer cells also adhered more strongly to culture dishes and led to decreased cell growth, but to a much lesser degree than with PJE. This data suggests a need for the interaction between all polyphenols found in PJE.

It is known that GI-1 is over-expressed in most cancers (15-17). In our study, we showed that pancreatic cancer cells treated with PJE decreased GI-1 expression, suggesting that PJE can inhibit the HH pathway. Based on the data, it is suggested that the polyphenols are interfering with the HH pathway by down regulating the GI-1 transcription factors. Since transcription of E-cadherin is inversely dependent on the GI-1 transcription factor, the decrease in the GI-1 expression increases the E-cadherin expression (16). While there is no published data to support that ezrin is directly regulated by GI-1, the similarities between E-cadherin and ezrin could suggest a similar interaction between GI-1 and ezrin in the HH signaling pathway. This relation is due to the fact that both are cell adhesion proteins and both have been shown to be involved in pancreatic cancer (19-22). We have demonstrated that both adhesion proteins have an increased expression when pancreatic cells were treated with PJE.

Our data contradicts the current studies on ezrin expression, including pancreatic cancer. In these studies, a high expression of ezrin correlates with a poor outcome (20-22). Specifically, ezrin leads to an increase in the spread of the cancer through its interaction with signaling events that are involved in the regulation of cell survival, proliferation, and migration (20-22). Like our data, a few studies have shown that weak ezrin expression correlates with poor patient outcome (24,25). Instead of pancreatic cancer, however, these studies examined serous ovarian carcinoma and lung adenocarcinoma (24,25). Our findings are the first to show that increased expression of ezrin leads to an inhibition of pancreatic cancer growth. Based on this study, we suggest that ezrin is regulated by the HH signaling pathway, similar to E-cadherin. When GI-1 levels are low, ezrin levels are high. In conclusion, this study suggests that PJE has possible benefits for pancreatic cancer. However, our experiments need to have increased replication to confirm these novel results.

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V. Gomez, T. Shackelford, A. Tocchi, M. Rowland-Goldsmith

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