Original Research Article

Punicalagin Restricts Growth, Promotes Apoptosis, and Reduces Invasion in Human Gastric Cancer Cells

Dose-Response:
An International Journal
April-June 2024:1–9

DOI: 10.1177/15593258241264954
journals.sagepub.com/home/dos
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Abstract

This research investigated the anticancer properties of punicalagin, a prominent bioactive polyphenol extracted from *Punica granatum* L, in human gastric cancer cell lines. Normal and gastric cancer cells were exposed to different doses of punicalagin for various durations. Punicalagin exhibited cytotoxic effects on gastric cancer cells in a dose- and time-dependent fashion, while sparing normal gastric epithelial cells. It is noteworthy that among the 3 gastric cancer cells, HGC-27 cells were more resistant to punicalagin than 23,132/87 and AGS cells. Furthermore, punicalagin triggered apoptosis in gastric cancer cells, evidenced by a rise in both early and late apoptotic cell percentages. Western blot analysis further revealed that punicalagin elevated the levels of activated caspase-3. Conversely, punicalagin curtailed cell invasion and reduced the expression of MMP-2, MMP-9, Snail, and Slug. From a mechanistic standpoint, Western blotting indicated that punicalagin might inhibit the Erk and NF-κB pathways, leading to apoptosis induction and the inhibition of cell invasion in gastric cancer cells. These results indicate that punicalagin promotes apoptosis and inhibits cell invasion in gastric cancer cells by activating caspase-3 and suppressing MMP-2, MMP-9, Snail, and Slug through the inhibition of the Erk and NF-κB pathways.

Keywords

punicalagin, gastric cancer, proliferation, apoptosis, cell invasion

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Received 5 March 2024; accepted 10 June 2024

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Introduction

Gastric cancer is the sixth most common cancer and the third leading cause of cancer deaths worldwide. Gastric cancer incidence has wide geographical variation, with low incidence in the Americas and high incidence in Africa, Europe, and Asia.[1,2] In 2021, gastric cancer was the eighth leading cause of cancer-related deaths in Taiwan.[3] Currently, the most common gastric cancer therapies include chemotherapy and gastrectomy. However, first-line drugs to treat gastric cancer in clinical practice are highly toxic and lack efficacy. Therefore, the development of less toxic and highly effective drugs remains a priority in the treatment of gastric cancer.

Punica granatum (pomegranate), due to its broad-spectrum health characteristics, has been used extensively as traditional medicine. For example, the pomegranate leaf exhibits antioxidant, anti-cancer and anti-inflammatory properties.[4] 2 types of polyphenols, anthocyanins and hydrolysable tannins, are found in pomegranate. Punicalagin is a hydrolysable tannin that is the main compound in pomegranate leaf and husk.[5] Punicalagin is documented to possess multiple beneficial properties, including antioxidant, anticancer, and anti-inflammatory effects.[6] Previous, including our studies have shown that punicalagin exerted therapeutic effects in various types of cancer including leukemia, osteosarcoma and colon cancer.[7-9] To the best of our understanding, the cytotoxic impact and the underlying mechanisms of punicalagin in gastric cancer have not been explored.

Apoptosis is a tightly controlled cellular death process that operates through 2 activation pathways: intrinsic and extrinsic.[10] It is activated during early embryonic development to eliminate unwanted cells. Apoptosis can be initiated through 1 of 2 pathways. The intrinsic pathway triggers cell death in response to cellular stress, while the extrinsic pathway is activated by signals from neighboring cells. Both pathways stimulate caspases, a group of endoproteases playing pivotal roles in regulating cell death within cellular networks, leading to cell death through the indiscriminate degradation of proteins. Additionally, apoptosis can serve as a mechanism to prevent cancer. The inhibition of apoptosis can lead to uncontrolled cell division and the subsequent development of tumors.

Epithelial-mesenchymal transition (EMT) is a biological process wherein epithelial cells transition from a polarized state, characterized by tight junctions, to a more migratory and invasive phenotype. EMT plays a vital role in facilitating cell-cell and cell-matrix interactions, as well as in the process of wound healing.[11,12] During EMT, epithelial cells alter their gene expression patterns, resulting in enhanced mobility and the attainment of invasive capabilities. However, neoplastic epithelial cells can deregulate EMT to accelerate cancer cell migration and invasion.[13]

Consequently, we utilized human primary gastric epithelial cells along with 3 distinct human gastric cancer cell lines (AGS, HGC-27, and 23,132/87) to investigate the anticancer properties of punicalagin. To the best of our knowledge, this research represents the first evidence that punicalagin displays cytotoxic effects specifically on gastric cancer cells without harming primary epithelial cells. Moreover, punicalagin prompted apoptosis via caspase-3 activation and inhibited the invasion of gastric cancer cells in vitro. The suppression of MMP-2, MMP-9, Snail, and Slug protein expression by punicalagin further underscored its anti-invasive capabilities.

Materials and Methods

Punicalagin

Punicalagin (2,3-(S)-hexahydroxydiphenoyl-4,6-(S,S)-gallagyl-D-glucose, CAS registry Number 65995-63-3, with a purity of ≥98%) was obtained from Sigma-Aldrich (Cat. No. P0023). It was dissolved in dimethylsulfoxide (DMSO; Sigma-Aldrich; Cat. No. D2650) to achieve a concentration of 50 mM and subsequently stored at −20°C until required.

Cell Culture and Treatments

Human primary gastric epithelial cells (purchased from Cell Biologics; Cat. No. H-6039) were maintained in Cell Biologics Complete Human Epithelial Cell Medium. Human gastric cancer cell line AGS (provided by the Food Industry Research and Development Institute; Cat. No. 60102) was grown in F-12K. Human gastric cancer cell line HGC-27 (provided by the European Collection of Cell Cultures; Cat. No. 94042256) was cultured in MEM. Human gastric cancer cell line 23 132/87 (provided by Creative Bioarray; Cat. No. CSC-C0324), were kept in RPMI-1640. Cells were cultured at 37°C in a 5% CO2 humidified environment. The cell lines underwent *mycoplasma* contamination checks and authentication by the respective cell providers. Additionally, we cross-referenced the ICLAC database to confirm the correct identification of all cell lines. The cell providers conducted *mycoplasma* tests for each cell line. Culture media for all cells comprised 10% fetal bovine serum (VWR, Cat. No. 89510-186), 100 units/mL penicillin G, 100 μg/mL streptomycin sulfate, and 250 ng/mL amphotericin B (all sourced from Thermo Fisher Scientific; Cat. No. 15240-062). This study involved treating cells with varying concentrations of punicalagin (ranging from zero to 500 μM) for durations of 24, 48, and 72 hr.

Cell Viability Assessment

Cell viability was determined using the CCK-8 assay kit (Biotools; Cat. No. TEN-CCK8), following the manufacturer's guidelines. The assay was conducted 3 times.

Apoptosis Assay

Apoptosis was analyzed using the Muse Annexin V and Dead Cell Kit (Merck Millipore; Cat. No. MCH100105), adhering to the manufacturer's instructions. The experiment was conducted in triplicate.

Caspase-3 Activity Assessment

Caspase-3 activity was assessed using a caspase-3 assay kit (Sigma-Aldrich; Cat. No. CASP3C). To suppress caspase-3 activity, the caspase-3 inhibitor N-acetyl-Asp-Glu-Val-Asp-CHO (Ac-DEVD-CHO; Biomol Research Laboratories; Cat. No. ABD-13403) was concurrently administered with punicalagin at a final concentration of 200 μM in a separate experiment. This analysis was conducted 3 times on separate occasions.

Cell Invasion Assay

The Cell Invasion Assay Kit (Merck Millipore; Cat. No. ECM550) was employed to evaluate cell invasion capabilities. The methodologies are elaborated upon in our prior publication.[14]

Western Blotting

Denatured protein samples were treated and loaded onto 15% SDS-PAGE gels, followed by transfer onto nitrocellulose membranes. After blocking, the membranes were incubated overnight at 4°C with the primary antibodies.: active caspase-3 (rabbit anti-human polyclonal, 1:100, Cat. No. AB3623, Merck Millipore), MMP-2 (mouse anti-human monoclonal, 1:1,000, Cat. No. NB200-114, Novus Biologicals), MMP-9 (mouse anti-human monoclonal, 1:1,000, Cat. No. NBP2-13173, Novus Biologicals), Snail (rabbit anti-human polyclonal, 1:1,000, Cat. No. NBP2-27184, Novus Biologicals), Slug (rabbit anti-human polyclonal, 1:1,000, Cat. No. ARG54888, Arigo Biolaboratories), Erk1/2 (rabbit anti-human monoclonal, 1:1000, Cat. No. 4695, Cell Signaling Technology), p-Erk1/2 (rabbit anti-human monoclonal, 1:2000, Cat. No. 4370, Cell Signaling Technology), IKKα (mouse anti-human monoclonal, 1:1000, Cat. No. 11930, Cell Signaling Technology), p-IKKα (rabbit anti-human monoclonal, 1:1000, Cat. No. 2697, Cell Signaling Technology), NF-κB (rabbit anti-human monoclonal, 1:1000, Cat. No. 8242, Cell Signaling Technology), and p-NF-κB (rabbit anti-human monoclonal, 1:1000, Cat. No. 3033, Cell Signaling Technology). After washing, membranes were incubated with peroxidase-conjugated secondary antibodies (goat anti-mouse/rabbit monoclonal, 1:20 000, Cat. No. A4416/A6154, Sigma) at room temperature for 1 hr. GAPDH (mouse anti-human monoclonal, 1:30 000, Cat. No. MAB374, Merck Millipore) served as the control to ensure consistent protein loading. Protein signals were visualized using enhanced chemiluminescence reagents (Thermo Fisher Scientific; Cat. No. 34096). The intensity of bands was assessed using GeneTools software (Syngene; Version 1.6.1.0).

Statistical Analysis

Unpaired Student's t test was used to identify significantly different means ($P < .05$ was considered to indicate a statistically significant difference).

Results

Punicalagin Induced Cell Death in Gastric Cancer Cells in Vitro

The cytotoxicity of punicalagin in AGS, HGC-27, and 23 132/87 gastric cancer cells was examined using CCK-8 assay. Treating AGS cells with varying concentrations of punicalagin (100, 200, 300, 400, and 500 μM) for durations of 24, 48, and 72 hr resulted in a decline in cell viability that was both concentration- and time-dependent compared to control cells (Figure 1B). The cell viability of human primary gastric epithelial cells did not significantly decrease with punicalagin treatment (Figure 1A). The IC_{50} value at 48 hr of punicalagin treatment was between 100 and 200 μM. A similar result was obtained after exposure of HGC-27 and 23 132/87 cells to punicalagin for 24, 48 and 72 hr (Figure 1(C) and (D)). A punicalagin concentration of 100 μM was selected for further experiments as significant cell death was noted at this concentration.

Punicalagin-Induced Apoptosis in AGS, HGC-27, and 23,132/87 Cells

In the initial phase of apoptosis, there's a disruption in cell membrane symmetry, leading to the externalization of phosphatidylserine (PS) residues within the plasma membrane. To delve deeper into whether the observed cytotoxic effects post-punicalagin treatment were attributable specifically to apoptosis, we utilized annexin V/PI double staining. This allowed us to quantify the proportion of early and late apoptotic cells in AGS, HGC-27, and 23,132/87 cell lines. Treatment with 100 μM punicalagin for 48 hr increased the apoptotic cell percentage across all gastric cancer cell lines (Figure 2). These findings suggest that the mechanism by which punicalagin induces cell death in AGS, HGC-27, and 23,132/87 cells is via the initiation of apoptosis.

Punicalagin Activated Caspase-3 in AGS, HGC-27, and 23,132/87 Cells

We investigated the involvement of caspase-3 in punicalagin-triggered apoptosis in AGS, HGC-27, and 23,132/87 cells. The findings revealed that after a 24-hour exposure to punicalagin, caspase-3 activity notably increased in all 3 cell lines (Figure 3A). To ascertain the significance of caspase-3 activation in punicalagin-mediated apoptosis, we examined the impact of the caspase-3 inhibitor, Ac-DEVD-CHO. Co-treatment of AGS cells with punicalagin and Ac-DEVD-CHO halted the activation of caspase-3 (Figure 3A). Western blot analysis, employing an anti-active caspase-3 antibody, further confirmed this enzymatic activity. Following a 48-hour punicalagin exposure, there was a heightened presence of active caspase-3, underscoring punicalagin's role in apoptosis via caspase-3 activation (Figure 3B). These findings suggest

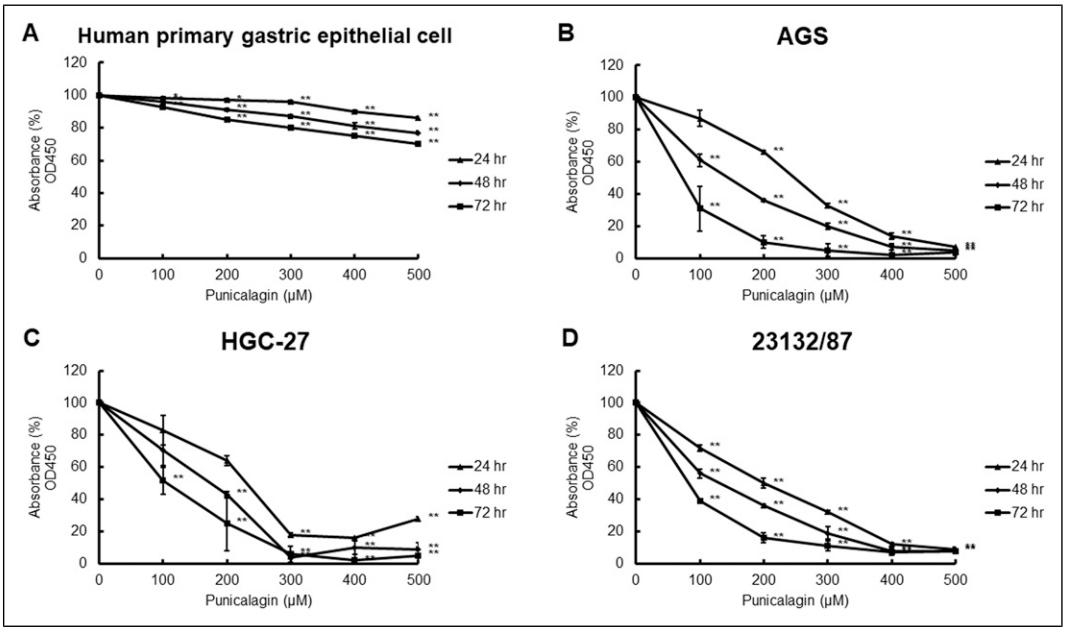

Figure 1. The impact of punicalagin on the survival of human gastric epithelial cells and gastric cancer cell lines was examined. Cells were exposed to varying concentrations of punicalagin for 24, 48, and 72 hr. Using a CCK-8 assay, absorbance was measured relative to the untreated control in (A) gastric epithelial cells, (B) AGS, (C) HGC-27, and (D) 23 132/87 cells. The data represent the mean ± S.D. from 3 separate experiments (*P < .05, **P < .01 compared to the DMSO control).

that caspase-3 activation is essential for punicalagin-induced apoptosis across these 3 cell lines.

Punicalagin Reduces the Invasion Capability of Gastric Cancer Cells

To assess punicalagin's impact on invasion, we conducted a cell invasion assay involving AGS, HGC-27, and 23 132/87 cells. The data revealed that after a 48-hour punicalagin treatment, a reduced number of cells invaded compared to the control group, indicating a suppression in cell invasion due to punicalagin exposure (Figure 4A). Subsequently, we utilized Western blotting with anti-MMP-2, MMP-9, Snail, and Slug polyclonal antibodies to probe the influence of punicalagin on these invasion-related proteins. The findings demonstrated that a 48-hour exposure to 100 μM punicalagin led to diminished expression levels of MMP-2, MMP-9, Snail, and Slug in gastric cancer cells (Figure 4B). Thus, the data suggest that punicalagin might inhibit gastric cancer cell invasion by reducing the expression of key proteins associated with this process.

Punicalagin Promotes Apoptosis and Inhibits Cell Invasion through the Erk and NF-κB Pathways in Gastric Cancer Cells

Erk and NF-κB pathways play vital roles in regulating multiple biological processes such as apoptosis and cell invasion. To determine whether the Erk and NF-κB pathways related to punicalagin were involved in the induction of apoptosis and suppression of cell invasion, Western blotting analysis was used to measure the changes of p-Erk1/2, p-IKKα and p-NF-κB levels. Compared to the control group, 48 hr treatment with punicalagin decreased the expression of p-Erk1/2, p-IKKα, and p-NF-κB in AGS, HGC-27 and 23 132/87 cells (Figure 5). The results indicate that punicalagin may interfere Erk and NF-κB pathways to induce apoptosis and suppress cell invasion in gastric cancer cells.

Discussion

Gastric cancer ranks as the third most common cause of cancer-related deaths worldwide, and more than 1 million new gastric cancer cases are reported annually.[15] Gastric cancer remains a significant health burden due to the low survival rate of patients with late stage disease.[16] Due to their high toxicity and low specificity, the standard treatments for gastric cancer (chemotherapy and gastrectomy) are largely ineffective.[17] Therefore, it is imperative to explore novel, less toxic treatments for this disease. The tannin punicalagin has been documented to exhibit therapeutic effects against various cancer types. Berköz et al demonstrated that punicalagin functioned as an antiproliferative agent specifically in A549 lung cancer cells, sparing normal lung cells.[18] Similarly, a separate study on leukemia cells revealed that punicalagin exhibited cytotoxic effects in AU565-PAR human breast cancer cells while leaving normal cells unaffected.[7] The cytotoxic activity of punicalagin in gastric cancer remains unknown. In our research, we demonstrated that punicalagin

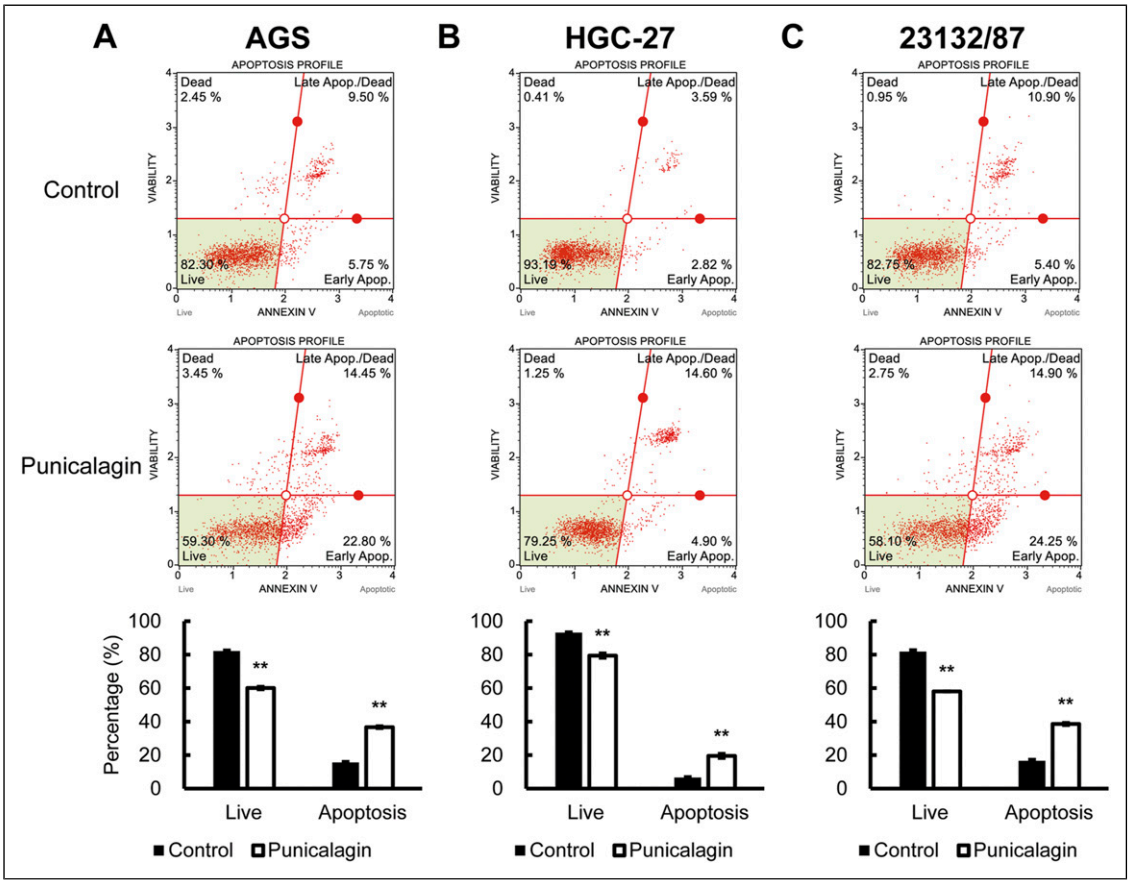

Figure 2. The impact of punicalagin on apoptosis in human gastric cancer cell lines was investigated. After treating cells with 100 μM punicalagin for 48 hr, they were assessed using annexin V/PI double staining. Treatment with punicalagin consistently resulted in an increased proportion of apoptotic cells in (A) AGS, (B) HGC-27, and (C) 23 132/87 cells. Flow cytometry analysis diagrams illustrating these changes are provided. The data represent the mean ± S.D. from 3 separate experiments (**$P < .01$ compared to the DMSO control).

displayed anticancer properties in AGS, HGC-27, and 23 132/87 gastric cancer cells without affecting normal gastric epithelial cells. These findings indicate that punicalagin could be a promising therapeutic agent for gastric cancer treatment.

The induction of apoptosis is 1 strategy utilized in anticancer therapies.[19] Many studies show that inducers of the Erk- and NF-κB-dependent apoptosis are potential therapeutic candidates for cancer. It was shown by Chen et al. that erianin, a natural compound isolated from Dendrobium, induced both apoptosis and autophagy by regulating MAPK signaling pathways in oral squamous cell carcinoma cells.[20] Celecoxib, a frequently prescribed antipyretic and analgesic medication, was discovered to enhance the apoptosis of liver cancer cells by reducing the expression of p-Erk.[21] It was reported that baicalein inhibited invasion and promoted apoptosis in glioma cells through the PI3K/Akt pathway.[22] Hyperoside, a flavonoid glycoside, could induce apoptosis of human lung cancer A549 cells through the ROS/p38 MAPK pathway.[23] Research conducted in osteosarcoma cells revealed that punicalagin suppressed osteosarcoma growth and metastasis by regulating NF-κB signaling. Studies with lung cancer A549 cells also showed that punicalagin initiated ROS-mediated apoptotic cell death through inhibiting STAT3 translocation.[24,25] Punicalagin also induced apoptosis through suppression of NF-κB and β-catenin signaling pathways in ME-180 cells.[26] However, the mechanism by which punicalagin triggers cell death in gastric cancer is still not fully understood. In this study, we demonstrated that punicalagin induced gastric cancer cell death through apoptosis, via the activation of caspase-3. Furthermore, Western blotting suggested that punicalagin might induce apoptosis by suppressing the phosphorylation of Erk, IKK, and NF-κB.

EMT, a reversible transition where epithelial cells shift between epithelial and mesenchymal states, is essential for typical development and tissue repair. Aberrant activation of EMT promotes increased migration and invasion capabilities, along with heightened resistance to chemotherapy. This is linked to the aggressive features of cancer cells during their progression and spread.[27,28] MMPs play a crucial role in normal development by contributing to cellular regeneration, programmed cell death, angiogenesis, and various other essential physiological processes. They also play roles in numerous pathological processes like EMT. Erk and NF-κB, responsible for transmitting intracellular signals, are involved in diverse

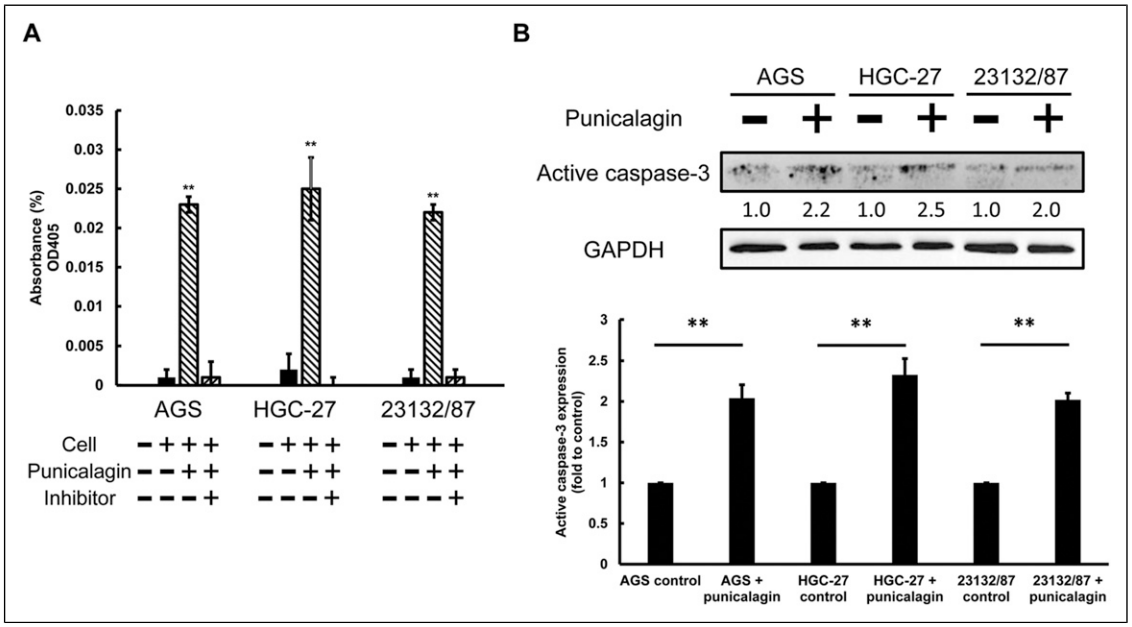

Figure 3. Punicalagin triggers caspase-3 activation in human gastric cancer cell lines. (A) Cells were treated with 100 μM punicalagin for 48 hr, either alone or in conjunction with a caspase-3 inhibitor, Ac-DEVD-CHO, which was added at a final concentration of 200 μM to suppress caspase-3 activity. Caspase-3 activity was quantified by measuring the release of p-nitroaniline (OD405), with each bar representing the activity level. The results, derived from 3 separate experiments, are shown as mean values ±S.D. (**$P < .01$ compared to the DMSO control). (B) Whole-cell lysates were harvested and analyzed via Western blotting using antibodies against active caspase-3 and GAPDH.

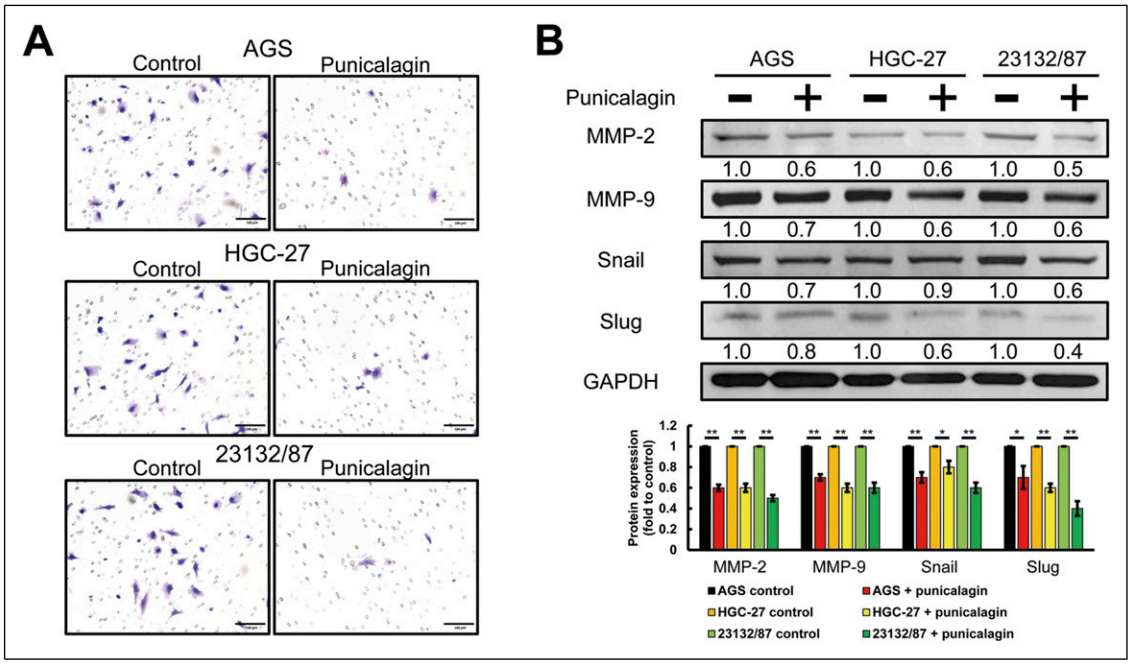

Figure 4. The impact of punicalagin on the invasiveness of human gastric cancer cell lines was studied. (A) Cells were exposed to 100 μM punicalagin for 48 hr, followed by assessment using a Cell Invasion Assay kit. The displayed data is representative of 3 distinct experiments. Scale bar = 100 μm. (B) Whole-cell lysates were obtained and analyzed via Western blotting, employing antibodies against MMP-2, MMP-9, Snail, Slug, and GAPDH.

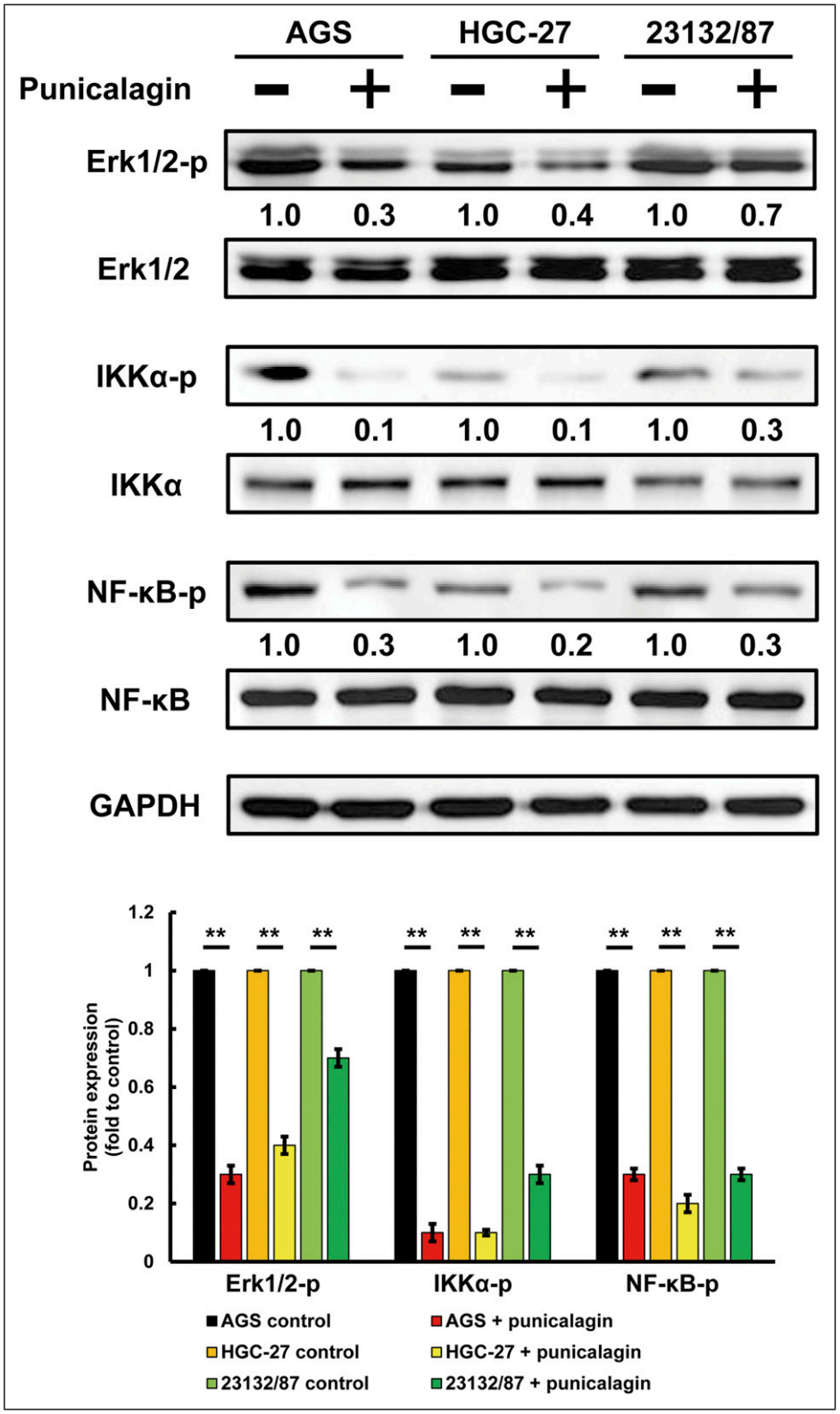

Figure 5. The influence of punicalagin on the Erk and NF-B signaling pathways in human gastric cancer cell lines was investigated. Cells were exposed to 100 μM punicalagin for 48 hr. Subsequently, whole-cell lysates were harvested and analyzed via Western blotting, utilizing antibodies against Erk1/2, p-Erk1/2, IKKα, p-IKKα, NF-κB, p-NF-κB, and GAPDH.

biological activities, including cell invasion. Compounds that inhibit Erk and NF-κB signaling pathways could be promising for cancer treatment. A recent study conducted by Chuang et al. indicated that a natural compound isolated from Danshen, salvianolic acid A, could suppress cell invasion through Erk pathway in nasopharyngeal carcinoma.[29] Scutellarin, a plant flavone, was found to inhibit cell invasion of leukemia cells by targeting the Erk pathway.[30] Jin and colleagues found that magnolol, a bioactive compound derived from roots and barks of different magnolia species, inhibited the invasion of myeloma cells by reducing the expression of p-NF-κB.[31] Opioid analgesic fentanyl could impair the invasion of glioma cells by modulating NF-κB activation.[32] The effect of punicalagin on cell migration and invasion has been described in several studies. Punicalagin was demonstrated to suppress osteosarcoma growth and metastasis by regulating NF-κB signaling[24] Punicalagin suppressed HeLa cell migration by decreasing MMP-2 and MMP-9 levels and increasing the expression of TIMP-2 and TIMP-3.[26] Pan et al. indicated that punicalagin reduced the migration and invasion abilities of breast cancer MCF-7 and MDA-MB-231 cells by elevating E-Cadherin levels and lowering MMP-2, MMP-9, and N-Cadherin levels.[33] Punicalagin inhibited the proliferation, invasion, and angiogenesis of osteosarcoma cells by disrupting the NF-κB signaling pathway.[8] Consistent with prior research, the findings of this study suggest that punicalagin inhibited the invasion of gastric cancer cells by reducing levels of MMP-2, MMP-9, Snail, and Slug.

In conclusion, this research revealed, for the first time, that punicalagin curbed gastric cancer growth by limiting both proliferation and invasion, offering initial evidence endorsing it as a promising and novel option for gastric cancer chemotherapy. Additional investigations are needed to pinpoint the specific therapeutic targets responsible for punicalagin's cytotoxic effects and its ability to hinder invasion.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

Funding

The authors disclose receipt of the following financial support for the research, authorship, and/or publication of this article: This work was supported by 2 grants (109CM-TMU-03, 111CM-TMU-04) from Chi Mei Medical Center.

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