Astragaloside IV promotes the apoptosis of pancreatic cancer cells by activating endoplasmic reticulum stress through the PERK/ATF4/CHOP signaling pathway

YIJUN WANG[1], MENGYANG ZHAO[1], SHANSHAN LIU[1], RUI ZHENG[2] and TIANHUI GAO[1]

[1]Department of Oncology, Henan Provincial People's Hospital, People's Hospital of Zhengzhou University, People's Hospital of Henan University, Zhengzhou, Henan 450001, P.R. China; [2]State Key Laboratory of Chemical Oncogenomics, Guangdong Provincial Key Laboratory of Chemical Genomics, Peking University Shenzhen Graduate School, Shenzhen, Guangdong 518071, P.R. China

Received May 30, 2025; Accepted October 17, 2025

DOI: 10.3892/mmr.2025.13748

Abstract. Pancreatic cancer is characterized by short survival and poor treatment outcomes. Astragaloside IV (AST-IV), the primary pharmacological component of *Astragalus membranaceus*, is a traditional Chinese medicinal component with demonstrated anticancer potential. The present study aimed to evaluate the therapeutic efficacy of AST-IV against pancreatic cancer cells *in vitro* and to elucidate its underlying mechanisms of action, thereby providing novel insights for its clinical application in the treatment of pancreatic cancer. The effects of AST-IV on PANC-1 pancreatic cancer cell viability and migration were assessed using Cell Counting Kit-8 and wound healing assays, respectively. Subsequently, RNA-sequencing (RNA-seq) analysis was performed, followed by Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses to investigate the mechanisms underlying the effects of AST-IV. Finally, western blotting experiments were conducted to validate the potential molecular mechanisms of AST-IV. The results revealed that AST-IV effectively suppressed the proliferation and migration of the pancreatic cancer cells. In addition, GO and KEGG analyses of the differentially expressed genes identified by RNA-seq analysis suggested that AST-IV induced endoplasmic reticulum (ER) stress and influenced critical cellular processes, including cell cycle regulation and DNA damage repair. Furthermore, western blotting demonstrated that AST-IV significantly activated the protein kinase R-like endoplasmic reticulum kinase (PERK) signaling pathway, upregulated activating transcription factor 4 expression and induced the overexpression of CCAAT/enhancer-binding protein homologous protein, indicating that it triggered apoptosis. In summary, these findings suggest that AST-IV induced apoptosis in pancreatic cancer cells through PERK-mediated ER stress. These results expand the potential therapeutic applications of AST-IV and provide a theoretical foundation for the development of novel treatment strategies and therapeutic targets for pancreatic cancer treatment.

Correspondence to: Professor Tianhui Gao, Department of Oncology, Henan Provincial People's Hospital, People's Hospital of Zhengzhou University, People's Hospital of Henan University, 7 Weiwu Road, Jinshui. Zhengzhou, Henan 450001, P.R. China
E-mail: gaotianhui123456@163.com

Key words: pancreatic cancer, astragaloside IV, endoplasmic reticulum stress, PERK signal pathway

Introduction

Pancreatic cancer is a highly aggressive and prognostically poor malignancy of the digestive system, which has been referred to as the 'king of cancers' (1) and is steadily increasing in prevalence worldwide (2,3). Due to its insidious onset and the lack of reliable early diagnostic and therapeutic strategies, most patients are diagnosed at an advanced stage, precluding curative surgical intervention (4). Consequently, chemotherapy remains the primary treatment modality for pancreatic cancer. The current recommended adjuvant chemotherapy regimen for patients with a good performance status is a combination of fluorouracil, oxaliplatin, irinotecan and leucovorin, known as modified FOLFIRINOX (5,6). For patients who exhibit a suboptimal response to gemcitabine- or capecitabine-based therapy, alternative options include DNA-damaging agents that interfere with DNA synthesis and repair, such as oxaliplatin and irinotecan, as well as antimetabolites, including gemcitabine and fluorouracil (7,8). However, the safety profiles of these agents and the development of drug resistance pose major clinical challenges. Therefore, the identification of effective therapeutic targets is critical for achieving an early diagnosis and improved prognosis for patients with pancreatic cancer.

Astragalus polysaccharides and astragalosides are the primary active constituents of *Astragalus membranaceus* (Radix Astragali, Huangqi), among which astragaloside IV (AST-IV; $C_{41}H_{68}O_{14}$; molecular weight, 784.97 Da) is the most biologically potent small compound (9). AST-IV is a tetracyclic triterpenoid saponin with a lanostane-type structure (Fig. 1A),

and AST-IV exhibits a broad spectrum of pharmacological activities. Notably, it displays anti-inflammatory, antioxidant, antifibrotic, antidiabetic, immunomodulatory and cardioprotective effects, mediated through the modulation of diverse signaling pathways (10-12). AST-IV has also been reported to exert therapeutic effects in various pathological conditions, including cerebral ischemia/reperfusion injury, pulmonary diseases, liver cirrhosis, cardiovascular disorders and diabetic nephropathy (13,14).

Studies have demonstrated the efficacy of AST-IV as an anticancer agent (13-16). Notably, Xu *et al* (15) reported that AST-IV effectively suppresses the proliferation and migration of lung cancer cells, and attenuates metastasis *in vivo*. In addition, a review by Tang *et al* (16) reported that AST-IV inhibits colorectal cancer cell proliferation, enhances immune responses and reduces drug resistance. Although these findings suggest that AST-IV has promising antitumor properties, its precise mechanisms of action remain unclear. Therefore, further investigation is required to elucidate the molecular pathways underlying these anticancer effects.

In the present study, the ability of AST-IV to inhibit the proliferation and migration of pancreatic cancer cells was evaluated, and RNA-sequencing (RNA-seq) analysis was performed to elucidate its mechanism of action. Furthermore, *in vitro* analyses were performed to evaluate the effect of AST-IV on protein kinase R-like endoplasmic reticulum kinase (PERK) signaling pathway activation, and the expression of activating transcription factor 4 (ATF4) and apoptosis-related proteins, including CCAAT/enhancer-binding protein homologous protein (CHOP). The overall aim of the study was to explore the potential of AST-IV as a candidate for pancreatic cancer treatment.

Materials and methods

Cell culture. The PANC-1 (cat. no. SCSP-535) human pancreatic cancer cell line was obtained from The Cell Bank of Type Culture Collection of The Chinese Academy of Sciences. Cells were cultured in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin (all Gibco; Thermo Fisher Scientific, Inc.), and maintained under sterile conditions at 37˚C in a humidified incubator with a 5% CO_2 atmosphere.

Drug. AST-IV (purity >99%; cat no. HY-N0431) was obtained from MedChemExpress (MCE). The compound was dissolved in dimethyl sulfoxide to prepare stock solutions of varying concentrations, which were subsequently diluted in culture medium to the desired working concentrations to treat the cells.

Cell viability assay. PANC-1 cells were detached using 0.25% trypsin (Gibco; Thermo Fisher Scientific, Inc.) and resuspended at a density of $1x10^4$ cells/ml. The cell suspension (100 μl/well) was seeded into 96-well plates, and the cells were allowed to adhere for 6 h under standard culture conditions. Following attachment, the cells were treated with AST-IV at concentrations of 0, 20, 40, 80, 100 and 200 μM for 24, 48 or 72 h, at 37˚C. Cell viability was then assessed using a Cell Counting Kit-8 (CCK-8; cat. no. HY-K0301; MCE). In brief, 10 μl diluted CCK-8 working solution was added to each well, and after gentle agitation, the plates were incubated at 37˚C for 30 min. The absorbance at 450 nm was measured using a microplate reader (Multiskan FC; Thermo Fisher Scientific, Inc.), and the cell viability was calculated accordingly.

Wound healing assay. The migratory capacity of PANC-1 cells was assessed using a wound healing assay. The cells were seeded in 12-well plates at a density of $1x10^5$ cells/well and cultured to 90-100% confluency. Following aspiration of the culture medium, wounds were created by scraping the cell monolayer with a sterile pipette tip in a perpendicular direction, after which dislodged cells were removed by gentle washing with PBS. The cells were then treated with serum-free medium containing 80 μM AST-IV. The width of the wound was documented at 0 h (baseline), and wound closure was evaluated at 24, 48 and 72 h post-treatment under a light microscope (Eclipse Ci-S; Nikon Corporation) using Image J software (v1.8.0, National Institutes of Health),

RNA-seq analysis. PANC-1 cells were seeded at a density of $1x10^6$ cells and cultured as aforementioned, followed by treatment with 80 μM AST-IV or an equivalent volume of DMSO for 48 h at 37˚C. Total RNA was extracted using TRIzol® Reagent (cat. no. 15596026CN, Invitrogen; Thermo Fisher Scientific. Inc.), and the concentration, quality and integrity of the RNA were quantified using a NanoDrop spectrophotometer (Thermo Fisher Scientific, Inc.). A total of 3 μg RNA per sample was used as an input for library preparation. Illumina Paired End Sample Prep kits (cat. no. 67700, Illumina, Inc.) were used to prepare libraries. Remaining overhangs were converted into blunt ends via exonuclease/polymerase activities and the enzymes were removed. After adenylation of the 3' ends of the DNA fragments, Illumina PE adapter oligonucleotides were ligated to prepare for hybridization. To select cDNA fragments of the preferred 400-500 bp in length, the library fragments were purified using the AMPure XP system (Beckman Coulter). Each cDNA library was sequenced using an Illumina Hiseq 4000 (cat. no. PE150; Illumina, Inc.). Sequencing analysis was performed by Shanghai Personal Biotechnology Co. Ltd. The raw data were processed and analyzed using the Personalbio GenesCloud online platform (genescloud.cn).

Read counts for each gene were quantified using HTSeq (v0.9.1, htseq.readthedocs.io/en/release_0.9.1/) to obtain the raw expression values. Gene expression levels were then normalized to fragments per kilobase of transcript per million fragments. Differential gene expression analysis was performed using DESeq2 (v1.38.3, bioconductor.org) with screening thresholds set at $|\log_2\text{FoldChange}| > 1$ and $P<0.05$. Bi-directional clustering analysis of all differentially expressed genes (DEGs) was conducted using the ComplexHeatmap (v2.16.0, https://www.bioconductor.org) software package. Clustering was based on the Euclidean distance and the complete linkage method, and heatmaps were generated to visualize gene expression patterns across samples.

Gene Ontology (GO) enrichment analysis was performed to identify the biological functions associated with the DEGs. The number of DEGs enriched in each term was calculated, and GO enrichment analysis was performed on the upregulated

and downregulated DEGs using ClusterProfiler (v4.6.0). P-values were calculated using the hypergeometric distribution method, with a threshold of P<0.05. Significantly enriched GO terms were used to determine the main biological functions, molecular functions and cellular components associated with the DEGs. Similarly, Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis of the DEGs was performed using ClusterProfiler (v4.6.0) software, with a threshold of P<0.05. Detailed results of these analyses are presented in Table SI. In addition, the RNA-seq data generated in the present study are all publicly available at the Sequence Read Archive (SRA) under accession number PRJNA1312337 (ncbi.nlm.nih.gov/sra/PRJNA1312337).

Western blot analysis. Western blotting was performed as previously described (17), with brief modifications. Cells were lysed in whole-cell lysis buffer (cat. no. P0013; Beyotime Institute of Biotechnology) and collected by scraping. The lysates were pelleted by centrifugation at 10,000 x g for 10 min at room temperature, and protein concentrations were determined using a BCA protein assay kit (cat. no. P0012; Beyotime Institute of Biotechnology). Equal amounts of protein (25 μg/lane) were separated by 10% SDS-polyacrylamide gel electrophoresis and transferred onto polyvinylidene difluoride membranes (MilliporeSigma). Membranes were blocked with 10% skimmed milk for 1 h at room temperature, then incubated overnight at 4˚C with the following primary antibodies (1:1,000 dilution): 78-kDa glucose-regulated protein (GRP78; cat. no. 80849-1-RR), PERK (cat. no. 20582-1-AP), phosphorylated (p-)PERK (cat. no. 82534-1-RR), ATF4 (cat. no. 81798-1-RR), CHOP (cat. no. 81462-1-RR) and Bax (cat. no. 50599-2-Ig) from Proteintech Group, Inc., and eukaryotic translation initiation factor 2 a (eIF2α; cat. no. 9722), p-eIF2α (cat. no. 3398) and Bcl-2 (cat. no. 15071) from CST Biological Reagents Co., Ltd. After washing, the membranes were incubated with horseradish peroxidase-conjugated secondary antibodies (anti-rabbit; cat. no. SA00001-2) or anti-mouse (cat. no. SA00001-1) IgG; 1:5,000 dilution) for 30-60 min at room temperature. Protein bands were visualized using an enhanced chemiluminescence detection system (Amersham; Cytiva) and quantitatively analyzed using ImageJ software (v1.8.0, National Institutes of Health).

Statistical analysis. All data are presented as the mean ± standard error of the mean. Statistical comparisons were performed using Student's t-test or one-way analysis of variance followed by post-hoc analysis with Tukey's test. P<0.05 was considered to indicate a statistically significant result. All statistical analyses were performed using GraphPad Prism 8 (Dotmatics).

Results

AST-IV inhibits the proliferation and migration ability of PANC-1 pancreatic cancer cells. The potential effect of AST-IV on PANC-1 pancreatic cell viability was evaluated through comprehensive concentration- and time-dependent CCK-8 analyses. Only high concentrations of AST-IV (100 and 200 μM) significantly reduced PANC-1 cell viability after a 24-h treatment (Fig. 1B). With extended exposure, all tested concentrations (20-200 μM) significantly reduced cell viability at 48 and 72 h, with an inhibitory trend apparent over time (Fig. 1B). Growth curve analysis determined that the half-maximal inhibitory concentration of AST-IV at 48 h was ~80 μM, which was selected as the standard treatment concentration for 48-h exposure in subsequent experiments. Wound healing assays demonstrated that 80 μM AST-IV significantly suppressed PANC-1 cell migration at all assessed timepoints (24, 48 and 72 h; Fig. 1C). Collectively, these findings indicate the anticancer potential of AST-IV, revealing its dual ability to suppress the proliferative and migratory capacities of pancreatic cancer cells.

RNA-seq and DEG analysis. To elucidate the molecular mechanisms underlying the effects of AST-IV on pancreatic cancer cells, comprehensive RNA-seq analysis was performed and DEGs were identified. As shown in Fig. 2A, AST-IV treatment induced substantial alterations in the transcriptome of the pancreatic cancer cells, with 1,906 DEGs identified relative to the control group, of which 944 were significantly upregulated and 962 were significantly downregulated. Hierarchical clustering analysis (Fig. 2B) revealed clear separation between the treatment groups and consistent gene expression patterns within replicates, confirming the reproducibility of the data. The expression profiles of all identified DEGs are provided in Table SI. This transcriptomic analysis establishes a solid foundation for subsequent functional investigations of the mechanism of action of AST-IV in pancreatic cancer cells.

Enrichment analysis of upregulated DEGs in the AST-IV group. To investigate the mechanistic basis of the effects of AST-IV on pancreatic cancer cells, a comprehensive functional enrichment analysis of significantly upregulated DEGs was performed using GO and KEGG pathway analyses. GO enrichment revealed three primary functional categories affected by AST-IV treatment: i) Endoplasmic reticulum (ER) stress response, encompassing processes including 'endoplasmic reticulum unfolded protein response', 'misfolded protein binding', 'response to unfolded protein', 'protein folding in endoplasmic reticulum', 'ATF6-mediated unfolded protein response', 'retrograde protein transport, ER to cytosol' and 'p-ERK-mediated unfolded protein response'; ii) protein activity regulation, including 'histone H2AXY142 kinase activity', 'protein tyrosine kinase activity' and 'Atg1/ULK1 kinase complex'; and iii) signal transduction processes, including 'intracellular signal transduction', 'regulation of TORC1 signaling' and 'calcitonin gene-related peptide receptor signaling pathway'. Additional enriched processes included 'response to oxidative stress', 'positive regulation of interleukin-4 production' and 'positive regulation of interleukin-5 production' (Fig. 3A).

KEGG pathway analysis demonstrated significant enrichment of upregulated genes in 'protein processing in endoplasmic reticulum', 'metabolic pathways' and 'apoptosis', with notable involvement of key signaling pathways including the 'p53 signaling pathway', 'PI3K-Akt signaling pathway' and 'MAPK signaling pathway' (Fig. 3B). These systematic analyses collectively suggest that AST-IV may accelerate pancreatic cancer cell apoptosis via the modulation of ER stress-related processes and associated signaling cascades.

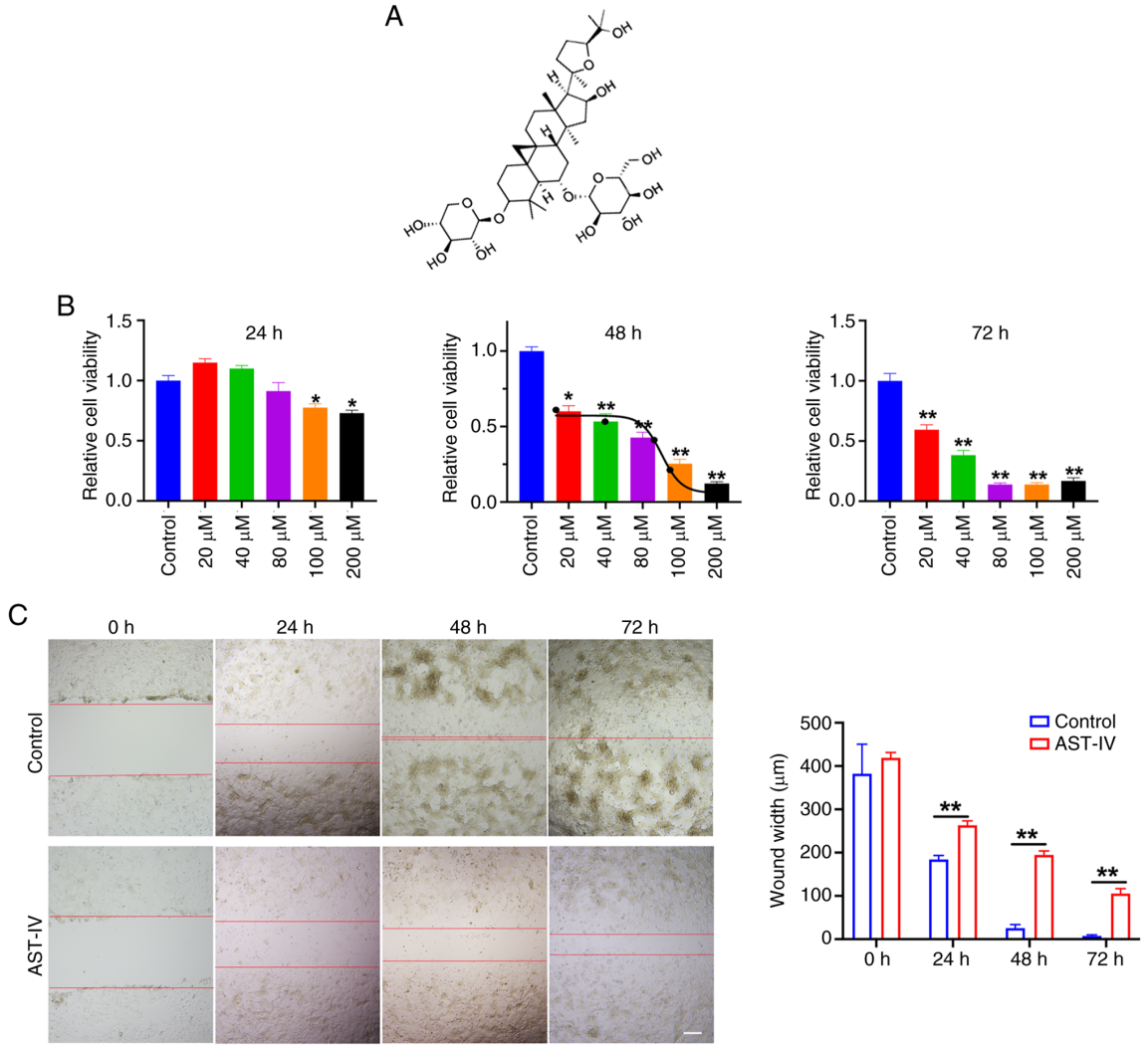

Figure 1. AST-IV inhibits the proliferation and migration of PANC-1 pancreatic cancer cells. (A) Chemical structure of AST IV. (B) Viability of PANC-1 cells detected via Cell Counting Kit-8 assay after treatment for 24, 48 and 72 h with various concentrations of AST-IV. A growth curve was also constructed after 48 h of AST-IV treatment. (C) Migration of PANC-1 cells detected by wound healing assay after treatment with 80 μM AST-IV for 24, 48 and 72 h. Scale bar, 100 μm. *P<0.05 and **P<0.01 vs. the control group. AST-IV, astragaloside IV.

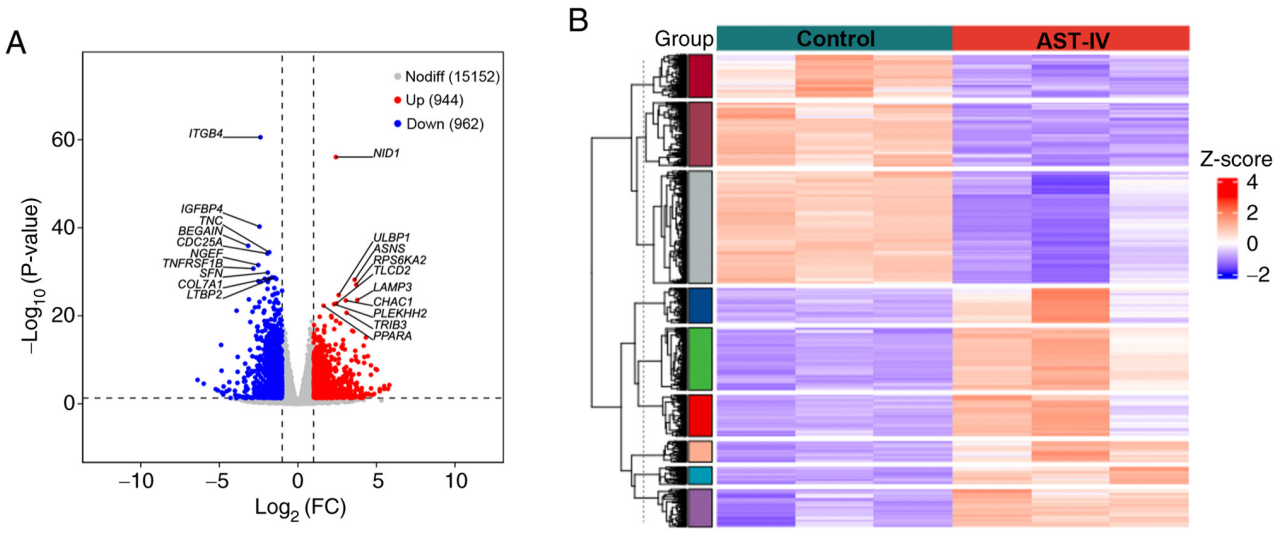

Figure 2. Volcano plot and heatmap of significant DEGs between the AST-IV treatment and control groups. (A) Volcano plot of the DEGs shows 1,906 transcripts that were significantly altered, with 944 upregulated (red) and 962 downregulated (blue) in the AST-IV group. (B) The DEGs were subjected to cluster analysis and the results are shown as heatmaps. AST-IV, astragaloside IV; DEGs, differentially expressed genes; FC, fold change; nodiff, no significant difference.

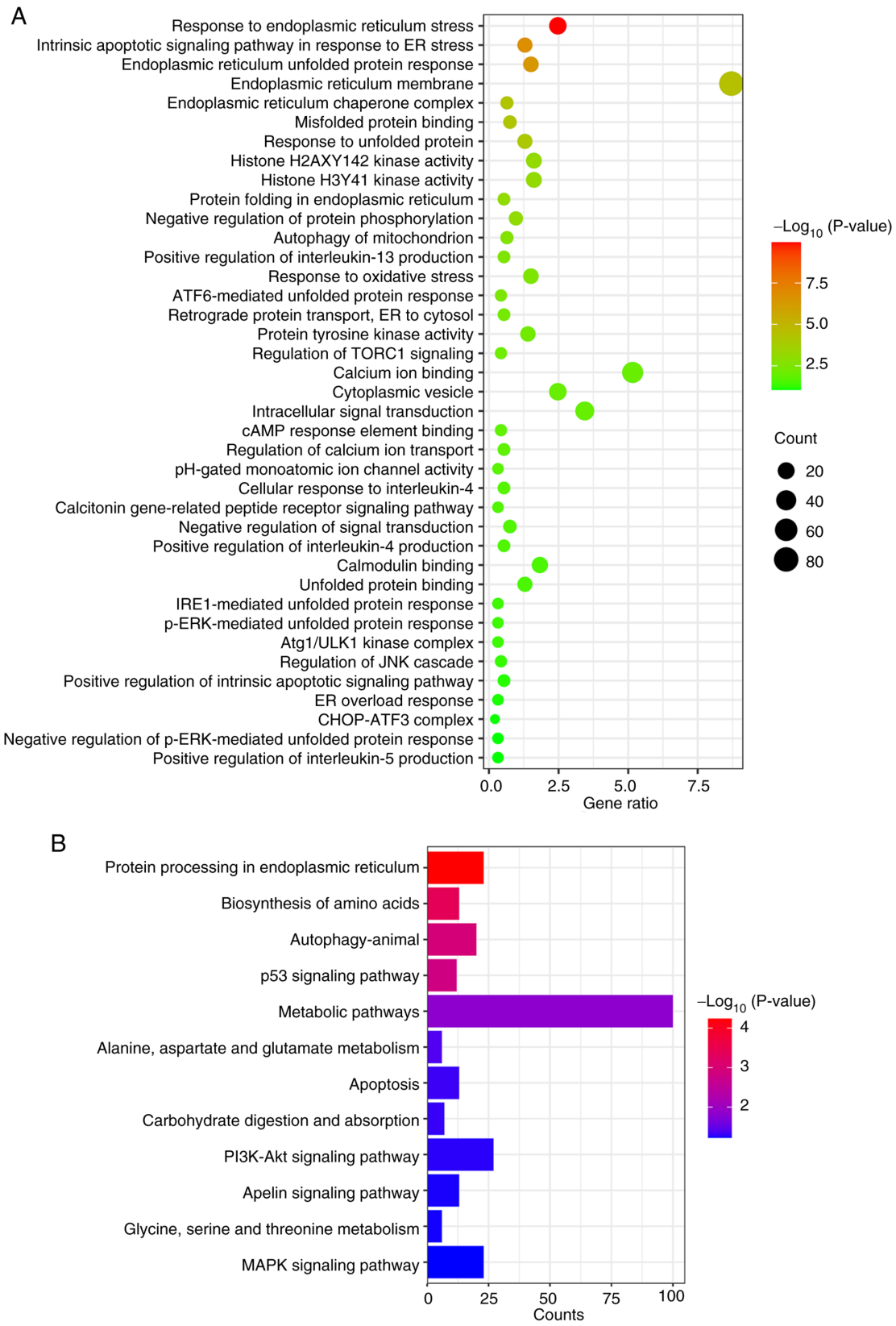

Figure 3. GO and KEGG enrichment analyses of upregulated DEGs in the AST-IV group compared with the control group. (A) GO enrichment of upregulated DEGs following treatment with 80 μM AST-IV for 48 h. (B) KEGG pathway enrichment analysis of upregulated DEGs under the same treatment conditions. AST-IV, astragaloside IV; DEG, differentially expressed genes; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes.

Enrichment analysis of downregulated DEGs in the AST-IV group. Similarly, comprehensive GO and KEGG enrichment analyses were conducted for the significantly downregulated genes following AST-IV treatment. GO analysis revealed two primary functional categories: i) Cell cycle-related processes, including 'G2/M transition of

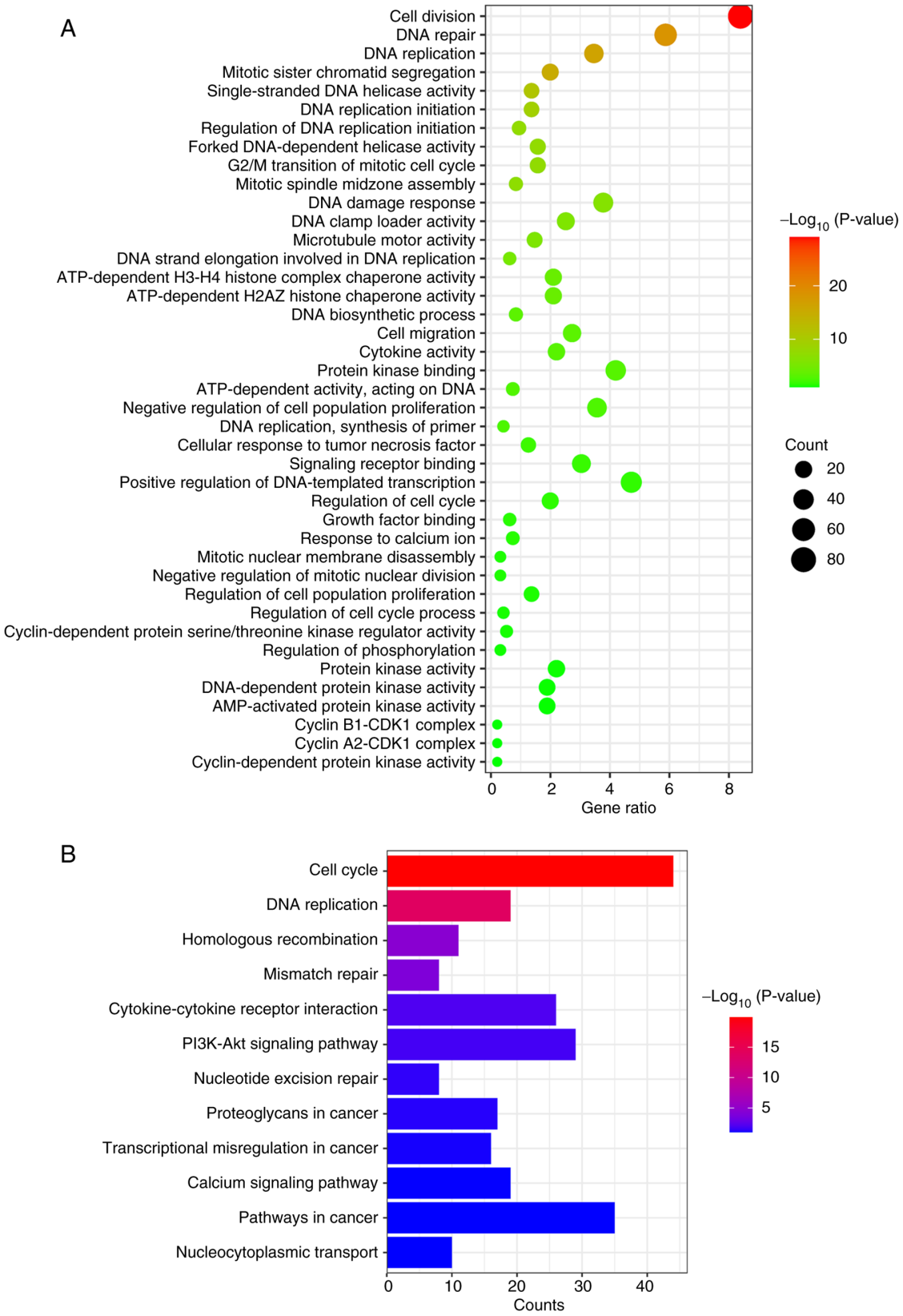

Figure 4. GO and KEGG enrichment analysis of downregulated DEGs in the AST-IV group compared with the control group. (A) GO enrichment of downregulated DEGs following treatment with 80 μM AST-IV for 48 h. (B) KEGG pathway enrichment analysis of downregulated DEGs under the same treatment conditions. AST-IV, astragaloside IV; DEGs, differentially expressed genes; GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes.

mitotic cell cycle', 'growth factor binding', 'cyclin-dependent protein serine/threonine kinase regulator activity', 'cyclin B1-CDK1 complex formation', 'cyclin-dependent protein kinase activity' and 'microtubule motor activity'; and ii) DNA damage processes, encompassing 'DNA repair', 'DNA replication', 'single-stranded DNA helicase activity',

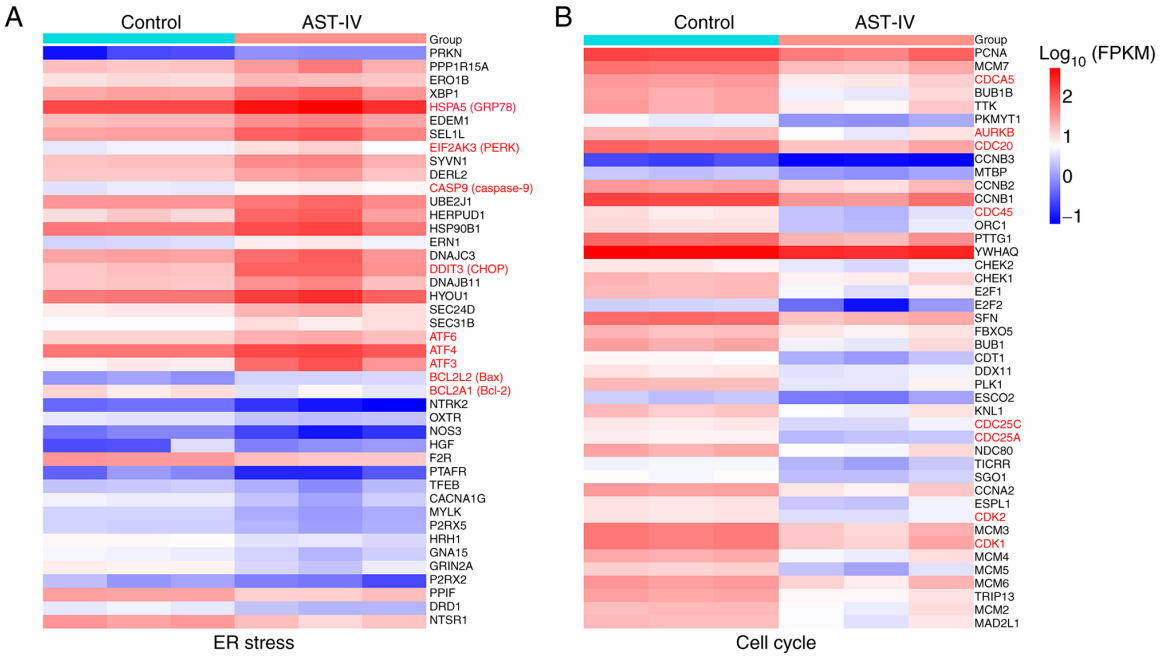

Figure 5. Analysis of the mechanism of AST-IV in the treatment of pancreatic cancer. Heatmap of differentially expressed marker genes involved in (A) ER stress and (B) the cell cycle. AST-IV, astragaloside IV; ER, endoplasmic reticulum; lg(FPKM), $\log_{10}$ (fragments per kilobase of transcript per million mapped fragments).

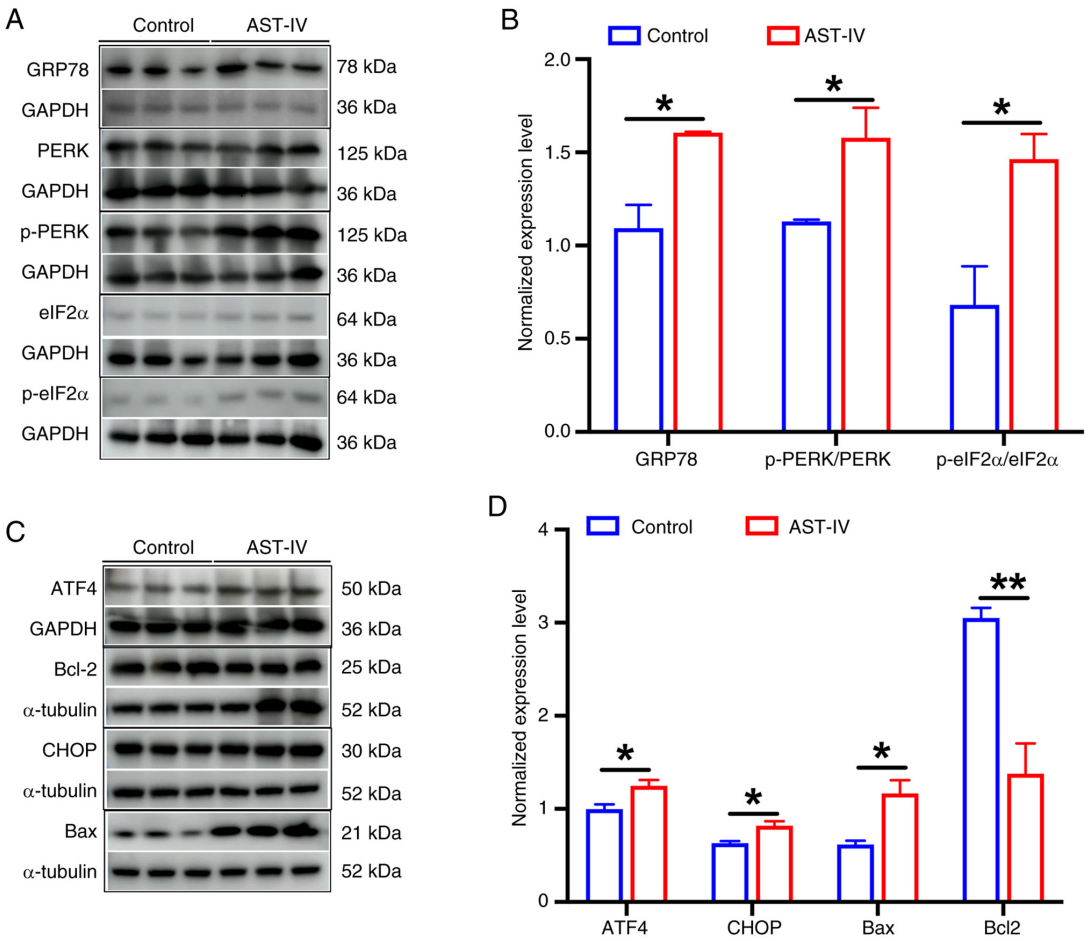

Figure 6. AST-IV activates the PERK signaling pathway and induces the apoptosis of pancreatic cancer cells. (A) Representative western blots of GRP78, PERK, p-PERK, eIF2α and p-eIF2α and (B) semi-quantitative analysis of the blots. (C) Representative western blots of ATF4, CHOP, Bcl-2 and Bax and (D) semi-quantitative analysis of the blots. Data are presented as the mean ± SEM (n=3). *P<0.05 and **P<0.01 as indicated. AST-IV, astragaloside IV; eIF2a, eukaryotic translation initiation factor 2 a; ATF4, activating transcription factor 4; CHOP, CCAAT/enhancer-binding protein homologous protein; GRP78, 78-kDa glucose-regulated protein; p-, phosphorylated; PERK, protein kinase R-like endoplasmic reticulum kinase.

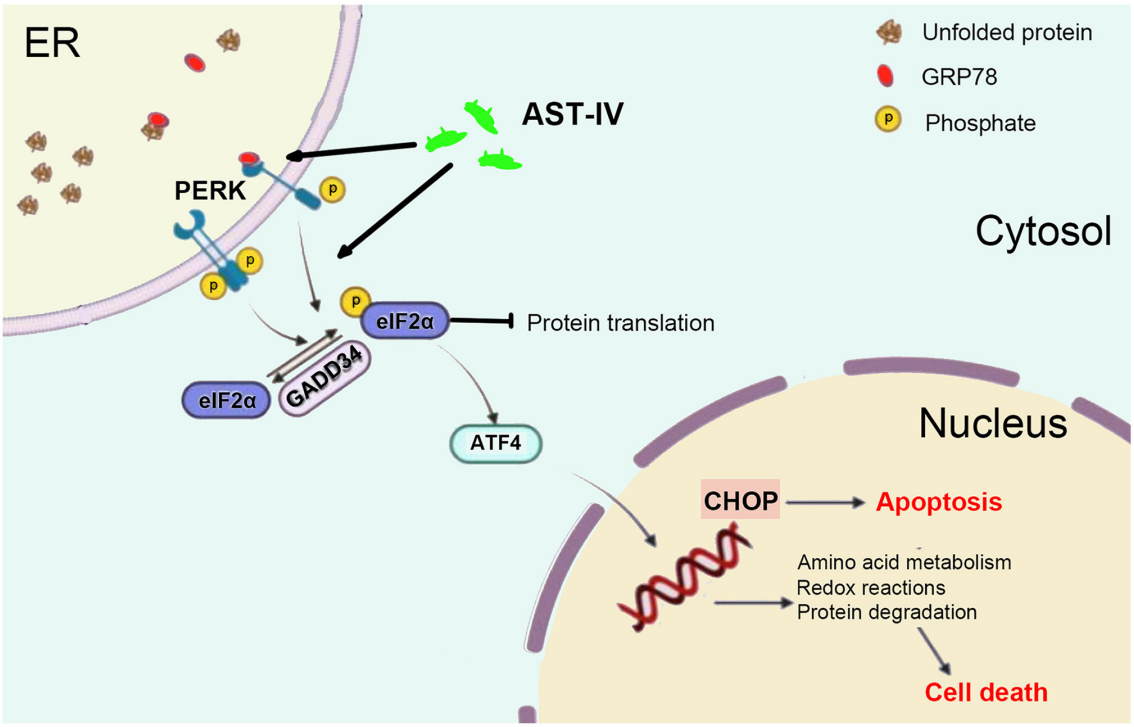

Figure 7. Schematic model illustrating the proposed mechanism by which AST-IV promotes apoptosis in pancreatic cancer cells. AST-IV induces ER stress via activation of the PERK signaling pathway, leading to upregulation of the downstream signaling factor ATF4. This subsequently upregulates the expression of pro-apoptotic proteins CHOP and Bax, while downregulating the anti-apoptotic protein Bcl-2, thereby promoting the apoptosis of pancreatic cancer cells. AST-IV, astragaloside IV; ATF, activating transcription factor; CHOP, CCAAT/enhancer-binding protein homologous protein; eIF2a, eukaryotic translation initiation factor 2 a; ER, endoplasmic reticulum; GADD34, growth arrest and DNA damage-inducible protein 34; GRP78, 78-kDa glucose-regulated protein; PERK, protein kinase R-like endoplasmic reticulum kinase; SP1/2, site-1/2 protease; UPR, unfolded protein response.

'forked DNA-dependent helicase activity', 'DNA clamp loader activity', 'DNA strand elongation involved in DNA replication' and 'DNA-dependent protein kinase activity'. Additional enriched processes included 'signaling receptor binding', 'AMP-activated protein kinase activity', 'cytokine activity' and 'protein kinase binding' (Fig. 4A).

KEGG pathway analysis demonstrated significant enrichment in cell cycle regulation and DNA damage repair pathways, along with 'homologous recombination', 'cytokine-cytokine receptor interaction', 'PI3K-Akt signaling pathway', 'nucleotide excision repair' and 'nucleocytoplasmic transport'. These systematic findings collectively indicate that AST-IV may exert its therapeutic effects against pancreatic cancer through the coordinated modulation of cell cycle progression and DNA damage response mechanisms.

Analysis of the mechanism of AST-IV in the treatment of pancreatic cancer. The comprehensive RNA-seq analysis revealed that AST-IV exerts its antitumor effects in pancreatic cancer through the coordinated regulation of ER stress and cell cycle pathways (Fig. 5A). The systematic evaluation of DEGs identified through GO and KEGG enrichment analyses demonstrated distinct molecular signatures associated with these processes. Key ER stress markers were significantly upregulated (Fig. 5A), including the molecular chaperone GRP78 [encoded by heat shock protein family A (Hsp70) member 5; *HSPA5*], the ER stress sensor PERK (eukaryotic translation initiation factor 2 a kinase 3; *EIF2AK3*), activating transcription factor 4 (*ATF4*) and the pro-apoptotic transcription factor CHOP (DNA damage-inducible transcript 3; *DDIT3*). This ER stress response was accompanied by the activation of downstream apoptotic effectors, including caspase 9 (encoded by *CASP9*) and Bax, indicating robust induction of programmed cell death (Fig. 5A).

Concurrently, marked downregulation of critical cell cycle regulator genes, including cell division cycle associated 5 (*CDCA5*), *CDC20*, *CDC45*, cyclin-dependent kinase 1 (*CDK1*) and *CDK2*, was observed, reflecting suppression of cell cycle progression (Fig. 5B). Collectively, these molecular patterns demonstrate that AST-IV has a dual mechanism of action, involving the simultaneous induction of ER stress-mediated apoptosis and suppression of cell cycle progression in pancreatic cancer cells.

AST-IV activates ER stress via the PERK signaling pathway to induce apoptosis in pancreatic cancer cells. Growing evidence indicates that ER stress is a critical driver of apoptotic cell death (18,19). Therefore, the ability of AST-IV to activate ER stress and the apoptotic pathway in pancreatic cancer cells was investigated. Treatment with AST-IV significantly upregulated the classical ER stress marker GRP78 (Fig. 6A and B), and the activation of PERK signaling, by significantly increasing p-PERK levels, while total PERK expression appeared to remain stable (Fig. 6A and B). This activation was propagated through downstream effectors, as evidenced by the significant elevation of p-eIF2α levels (Fig. 6A and B) and ATF4 expression (Fig. 6C and D). In addition, CHOP, recognized as a pivotal pro-apoptotic transcription factor in ER stress-mediated

apoptosis (20), exhibited significantly upregulated protein expression following AST-IV exposure (Fig. 6C and D). By contrast, the expression of the anti-apoptotic protein Bcl-2, a key regulator of cell survival in multiple malignancies (21), was significantly suppressed (Fig. 6C and D), while that of its pro-apoptotic counterpart Bax was significantly upregulated (Fig. 6C and D), indicating activation of the apoptotic pathway. Collectively, these findings demonstrate that AST-IV initiates ER stress, activates the PERK pathway, and promotes apoptosis through the coordinated regulation of both pro-death and pro-survival factors in pancreatic cancer cells. The sequential activation from early ER stress to downstream apoptotic effectors is suggested as a comprehensive mechanism underlying the cytotoxic activity of AST-IV.

Discussion

Recent epidemiological data from the United States identify pancreatic cancer as the fourth leading cause of cancer-related mortality, with projections indicating it may become the second most lethal malignancy by 2030 (22). The current therapeutic landscape remains largely centered on gemcitabine as the first-line chemotherapeutic standard; however, it has limited efficacy and the development of resistance inevitably occurs, which compromises long-term treatment outcomes (23,24). Although the combination of gemcitabine with nab-paclitaxel has emerged as a clinically meaningful advancement for advanced disease, it provides only marginal survival benefits, typically measured in weeks rather than months or years (25,26). This stark therapeutic gap highlights the critical unmet requirement for novel treatment strategies that can meaningfully improve patient outcomes.

In this context, natural product-derived compounds present a compelling alternative to conventional cytotoxic agents, offering comparable antitumor efficacy with potentially improved safety profiles. Among these, AST-IV has emerged as a particularly promising candidate due to its diverse biological activities and documented anticancer properties (27,28). The present aimed to characterize the pharmacological effects of AST-IV and to deepen the understanding of pancreatic cancer biology, with the ultimate goal of establishing an innovative therapeutic approach grounded in rigorous scientific evidence.

The results of the present study demonstrate that AST-IV effectively inhibits the proliferation and migration of pancreatic cancer cells, confirming its therapeutic potential against this malignancy. These findings are consistent with previous reports showing that AST-IV induces apoptosis via the mitochondrial-dependent intrinsic pathway and death receptor-dependent extrinsic pathway in various cancers, including colorectal cancer, breast cancer, lung cancer, vulvar squamous cell carcinoma and hepatocellular carcinoma (29-31), primarily by increasing the Bax/Bcl-2 ratio. Furthermore, Xia *et al* (32) reported that AST-IV exerts anticancer effects on SiHa cervical cancer cells by modulating autophagy-related proteins, including microtubule-associated protein 1 light chain 3, ubiquitin-like modifier-activating enzyme ATG7 and ubiquitin-like protein ATG12. In addition, AST-IV has been shown to suppress angiogenesis and reduce migration in HepG2 lung cancer cells by inhibiting the cyclooxygenase-2/prostaglandin E2/vascular endothelial growth factor axis (33,34). Distinct from these established mechanisms, the RNA-seq analysis performed in the current study revealed that AST-IV activates ER stress and induces apoptosis in PANC-1 pancreatic cancer cells, a previously unidentified mechanism underlying the anticancer activity of AST-IV. This novel insight expands our understanding of the multifaceted therapeutic potential of AST-IV beyond the established pathways observed in other malignancies.

The ER represents is a vital organelle that regulates cellular stress responses and serves as the primary site for protein synthesis, folding, transport and intracellular calcium storage (35,36). Both physiological and pathological stimuli can disrupt calcium homeostasis and alter protein folding, leading to the accumulation of misfolded proteins within the ER lumen and subsequent ER stress (37). Upon activation, ER stress signals are transduced across the ER membrane to the nucleus, initiating a cascade of molecular events collectively known as the unfolded protein response (UPR), a complex signaling network that exerts dual effects by either restoring cellular homeostasis or triggering apoptosis (37). ER stress-mediated apoptosis constitutes a distinct mechanism of cell death that differs from mitochondrial and death receptor-mediated pathways. This pathway is activated via three principal signaling branches, namely the PERK, inositol-requiring enzyme 1 (IRE1) and ATF6 signaling pathways (38). Under physiological conditions, these transmembrane sensors remain inactive through their association with the chaperone protein GRP78. During ER stress, GRP78 dissociates from these sensors to bind misfolded proteins, thereby allowing the activation of PERK and IRE1 by autophosphorylation (39). This initiates the UPR cascade and subsequent regulation of downstream transcription factors, ultimately leading to ER stress-induced apoptosis.

When cells accumulate excessive unfolded or misfolded proteins, GRP78 dissociates from PERK to preferentially bind these aberrant polypeptides (40). This dissociation triggers PERK activation, which subsequently phosphorylates eIF2α, resulting in a global attenuation of protein synthesis, serving as a crucial adaptive mechanism that alleviates ER protein loading and maintains metabolic homeostasis (41). The protein growth arrest and DNA damage-inducible protein 34 facilitates the dephosphorylation of eIF2α during ER-stress in cells (42). Paradoxically, p-eIF2α enhances the translation of ATF4, which upregulates the pro-apoptotic factor CHOP (43,44). Under conditions of persistent or unresolved ER stress, this PERK/ATF4/CHOP signaling axis becomes fully activated, ultimately committing the cells to apoptosis (45).

The RNA-seq analysis performed in the present study revealed the significant upregulation of key ER stress markers, including the molecular chaperone GRP78 (*HSPA5*), ER stress sensor PERK (*EIF2AK3*), *ATF4* and the pro-apoptotic transcription factor CHOP (*DDIT3*). These findings were confirmed at the protein level by western blotting. The AST-IV treatment significantly upregulated the expression of GRP78 and activation of PERK in the pancreatic cancer cells, accompanied by a marked elevation of downstream ATF4 expression, collectively indicating the induction of sustained ER stress.

CHOP is a pivotal pro-apoptotic transcription factor in ER stress-mediated apoptosis, which integrates signals

from all three ER stress sensors. While CHOP expression is tightly regulated at the transcriptional level in normal cells, accumulation of CHOP protein was observed in PANC-1 cells following AST-IV treatment, indicating the activation of irreversible apoptotic signaling via downstream effector regulation.

AST-IV was also observed to trigger the Bax/Bcl-2 pathway. Bax, a water-soluble protein homologous to Bcl-2 and a pro-apoptotic member of the Bcl-2 gene family, functions as a promoter of apoptosis (46). By contrast, Bcl-2 is embedded in the mitochondrial membrane where it sequesters Bax, thereby inhibiting the initiation of apoptosis. The overexpression of Bax can counteract the effect of Bcl-2, predisposing cells to apoptosis (47). The ratio of Bax to Bcl-2 is a well-established determinant of the balance between pro- and anti-apoptotic signaling (46,47). In the present study, AST-IV effectively promoted Bax expression and concurrently suppressed Bcl-2 expression, providing substantial evidence that AST-IV induces apoptosis in pancreatic cancer cells.

Due to technical limitations, although the present study identified the PERK signaling pathway as a mediator of AST-IV-induced ER stress, the direct molecular target(s) of AST-IV remain unclear and require further investigation. Furthermore, as the development and progression of pancreatic cancer are influenced by multiple factors within the *in vivo* microenvironment, future studies should include animal experiments to facilitate the drug development progress of AST-IV. Collectively, the data support the proposed mechanism (Fig. 7), in which AST-IV initiates persistent ER stress in pancreatic cancer cells, leading to activation of the PERK/ATF4/CHOP axis and culminating in caspase-mediated apoptosis. This represents a comprehensive ER stress-driven pathway distinct from the previously reported mechanisms of AST-IV in other malignancies.

Acknowledgements

The authors would like to thank Dr Yujie Zhang (Peking University, Beijing, China) for discussions and critical reading of the manuscript.

Funding

The present study was supported by the Science and Technology Research and Development Program Project of Henan Province (grant no. 192102310382).

Availability of data and materials

The data generated in the present study may be requested from the corresponding author. The RNA-seq data generated in the present study may be found in the Sequence Read Archive under accession number PRJNA1312337 or at the following URL: (ncbi.nlm.nih.gov/sra/PRJNA1312337).

Authors' contributions

YW, MZ, SL, RZ and TG contributed to study design. YW, MZ and SL and TG analyzed data. MZ, SL and RZ performed experiments. The first draft of the manuscript was written by YW and RZ, and TG revise the manuscript. YW, MZ, SL, RZ and TG confirm the authenticity of all the raw data. All authors read and approved the final version of the manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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