

Original Research

Activation of the MEK/ERK Pathway Mediates the Inhibitory Effects of Silvestrol on Nasopharyngeal Carcinoma Cells via RAP1A, HK2, and GADD45A

Lu-Rong Yu¹, Xian-Zhong Han^{2,*}, Ying-Zi Tang^{3,*}, Dan Liu², Xian-Qin Luo¹,
Xue-Wen Qiu², Jie Feng², Wen-Xiao Yuan², Jia-Yu Ding²

¹College of Traditional Chinese Medicine, Chongqing Medical University, 401331 Chongqing, China

²Department of Pharmacy, Chongqing General Hospital, Chongqing University, 400014 Chongqing, China

³Department of Pathology, Chongqing General Hospital, Chongqing University, 400014 Chongqing, China

*Correspondence: hxz5981@163.com (Xian-Zhong Han); hgltyz@163.com (Ying-Zi Tang)

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Abstract

Background: Nasopharyngeal carcinoma (NPC) is a malignant tumor associated with Epstein-Barr virus (EBV) infection. Chemoradiotherapy is the mainstream treatment for locally advanced NPC, and chemotherapeutic drugs are an indispensable part of NPC treatment. However, the toxic side-effects of chemotherapy drugs limit their therapeutic value, and new chemotherapy drugs are urgently needed for NPC. Silvestrol, an emerging natural plant anticancer molecule, has shown promising antitumor activity in breast cancer, melanoma, liver cancer, and other tumor types by promoting apoptosis in cancer cells to a greater extent than in normal cells. However, the effects of silvestrol on NPC and its possible molecular mechanisms have yet to be fully explored. **Methods:** Cell counting kit-8 (CCK-8), cell scratch, flow cytometry, 5-ethynyl-2'-deoxyuridine (EdU), and Western blot (WB) assays were used to evaluate the effects of silvestrol on the cell viability, cell cycle, apoptosis, and migration of NPC cells. RNA sequencing (RNA-Seq) was used to study the effect of extracellular signal-regulated kinase (ERK) inhibitors on the cell transcriptome, and immunohistochemistry (IHC) to assess protein expression levels in patient specimens. **Results:** Silvestrol inhibited cell migration and DNA replication of NPC cells, while promoting the expression of cleaved caspase-3, apoptosis, and cell cycle arrest. Furthermore, silvestrol altered the level of ERK phosphorylation. The ERK-targeted inhibitor LY3214996 attenuated silvestrol-mediated inhibition of NPC cell proliferation but not migration. Analysis of RNA-Seq data and WB were used to identify and validate the downstream regulatory targets of silvestrol. Expression of GADD45A, RAP1A, and hexokinase-II (HK2) proteins was inhibited by silvestrol and LY3214996. Finally, IHC revealed that GADD45A, RAP1A, and HK2 protein expression was more abundant in cancer tissues than in non-tumor tissues. **Conclusions:** Silvestrol inhibits the proliferation of NPC cells by targeting ERK phosphorylation. However, the inhibition of NPC cell migration by silvestrol was independent of the Raf-MEK-ERK pathway. RAP1A, HK2, and GADD45A may be potential targets for the action of silvestrol.

Keywords: nasopharyngeal carcinoma; silvestrol; apoptosis; ERK1/2; RAP1A; HK2; GADD45A

1. Introduction

Nasopharyngeal carcinoma (NPC) is a malignant tumor that develops in the upper part of the throat behind the nose. It has a distinct geographical distribution and is more common in East and Southeast Asia. The incidence of NPC in China ranges from 15 to 50 cases per 100,000 population and shows a decreasing trend from south to north due to environmental and genetic factors [1–3]. The incidence is slightly higher in males than females [4,5], and individuals with a positive family history have a four-fold increased risk of developing NPC [6].

The incidence of NPC is associated with Epstein-Barr virus (EBV) infection [7]. However, as of my last update, vaccines against EBV infection have shown potential but have not been widely implemented or shown to significantly reduce the incidence of NPC. Over the past decade, radiotherapy and chemoradiotherapy have been the

main treatments for NPC. Radiotherapy is the primary treatment for early-stage NPC as it is highly sensitive to ionizing radiation. However, radiotherapy alone has limited efficacy in T2N1 patients, and early treatment of these patients should be combined with chemotherapy [8]. For stage I-II NPC, intensity-modulated radiation therapy (IMRT) with or without chemotherapy has achieved a 5-year survival rate of >90% [9]. Chemoradiotherapy is the mainstream treatment for locally advanced NPC [10–12], and chemotherapeutic drugs are an indispensable part of NPC treatment [13,14]. However, the toxic side-effects of chemotherapy drugs limit their therapeutic value for NPC, and hence new chemotherapy drugs are urgently needed.

Silvestrol, a natural plant-derived anticancer molecule, has shown promising antitumor activity in breast cancer [15], melanoma [16], liver cancer [17], and other tumor types. It promotes a greater extent of apoptosis in cancer cells compared to normal cells [18]. Singh *et*



al. [19] demonstrated that silvestrol binds specifically to eIF4A and selectively inhibits the translation of GQ-enriched messenger RNAs (mRNAs) such as KRAS and its downstream signaling molecules. eIF4A plays a role in various cellular processes, and silvestrol exhibits varying effectiveness in different cancer types. This study aims to uncover the specific anti-tumoral effects and molecular mechanisms of silvestrol in NPC cells, with the ultimate goal of discovering new treatments for NPC patients.

2. Materials and Methods

2.1 Cell Culture

The human NPC cell lines HONE1 (Mingzhoubio, MZ-1647, Ningbo, China) and CNE2 (Immocell, IM-H136, Xiamen, China) were cultured in DMEM (Procell, PM150210, Wuhan, China) with 10% fetal bovine serum (Procell, 164210-500, Wuhan, China) and 1% penicillin-streptomycin double antibiotic (Beyotime, C0222, Shanghai, China) in a cell incubator at 37 °C and 5% CO₂. All cell lines were validated by STR profiling and tested negative for mycoplasma.

2.2 5-ethynyl-2'-deoxyuridine (EdU)-Based Cell Proliferation Assay

BeyoClick™ EdU-488 (Beyotime, C0071S, Shanghai, China) was used for the proliferation assay. HONE1 and CNE2 cells were seeded in 96-well plates (5×10^4 cells/well) and treated with silvestrol (Medchemexpress, HY-13251, Monmouth Junction, NJ, USA) after 24 h of culture. After another 24 h, EdU was added to each well and the cells kept at 37 °C for 2 h. Cells were then fixed with 4% paraformaldehyde (Biosharp, BL539A, Hefei, China) for 30 minutes, and immunofluorescence microscopy (Leica, DMI8, Wetzlar, Germany) was used to observe and record images. The percentage of EdU-positive cells was calculated by dividing the number of EdU-positive cells by the total DAPI-stained cell count. The ratio for the control group was set to 1 for reference.

2.3 Wound Healing Assay

Cells were cultured for 24 h until 90% confluence was achieved. The monolayer was scratched with the tip of a pipet gun and the exfoliated cells removed. Cells were then cultured in serum-free medium containing silvestrol with or without LY3214996 (Medchemexpress, HY-101494, Monmouth Junction, NJ, USA). The cell migration rate was calculated as follows: (Initial Scratch Width – Scratch Width after 24 h)/Initial Scratch Width $\times$ 100%

2.4 Apoptosis Assay

A total of 1.5×10^5 cells/well were seeded in 6-well plates. After 24 h, silvestrol with or without LY3214996 was added to the medium of the experimental group. Cells were then harvested by trypsin (Beyotime, C0207, Shanghai, China) digestion and washed with PBS. Staining

was performed using the Annexin V-FITC/PI apoptosis kit (MULTISCIENCES, 70-AP101-100, Hangzhou, China), and Annexin V-FITC and propidium iodide (PI) signals were measured by flow cytometry (Beckman, CytoFLEX LX, Miami, FL, USA).

2.5 Cell Cycle Assay

Silvestrol with or without LY3214996 was added to the medium of the experimental group. Trypsin digestion was carried out to harvest cells, followed by PBS washing and subsequent fixation in 75% alcohol for 24 h. Staining procedures were performed with PI/RNase Staining Buffer (BD Biosciences, 550825, San Diego, CA, USA).

2.6 Western Blotting

Cells were lysed in RIPA lysis buffer (Beyotime, P0013J, Shanghai, China) and 1% PMSF (Solarbio, P0100, Beijing, China) and then centrifugated. The extracted proteins were denatured with SDS-PAGE protein loading buffer (Beyotime, P0015, Shanghai, China) (100 °C, 10 min) and separated by 11% SDS-PAGE gel electrophoresis (Beyotime, P0903, Shanghai, China). Proteins were then transferred onto PVDF membranes (Beyotime, FFP22, Shanghai, China) and blocked with a 5% BSA (Beyotime, ST023, Shanghai, China) solution. Incubation with primary antibodies was carried out overnight at 4 °C, followed by subsequent incubation with horseradish peroxidase-labeled secondary antibodies for 1.5 h. The ultra-sensitive multifunctional imager (Cytiva, Amersham ImageQuant 800, Shanghai, China) was used to visualize protein bands. The following antibodies (Affinity, Wuhan, China) were used: β -actin (Affinity, AF7018), Cleaved Caspase3 (Affinity, AF7022), p-ERK1/2 (Affinity, AF1015), ERK1/2 (Affinity, AF0155), p-MEK (Affinity, AF8035), MEK (Affinity, AF6385), GADD45A (Affinity, DF6622), RAP1A (Affinity, DF6157), HK2 (Affinity, DF6176), and Goat Anti-Rabbit IgG (H+L) HRP (Affinity, S0001). We employed Image J (NIH, Pro Plus 6.0, MD, USA) to quantify the band intensities. The relative density was calculated by dividing the density of the target band by the density of β -actin. The relative density of the control group was set to 1 for normalization purposes.

2.7 IHC

The age range of the patients providing the pathological tissue sections is 50-60 years old. The providers of tissues #1 and #3 are male, while the provider of tissue #2 is female. Tissue sections (4 μ m) (Collection date: #1 - June 2022, #2 - November 2022, #3 - May 2022) were incubated at 60 °C for 20 minutes before undergoing xylene (Biofount, 1330-20-7, Beijing, China) deparaffinization and ethanol rehydration. Antigen retrieval was performed in 10 mM citric acid solution (Beyotime, P0081, Shanghai, China) and involved microwave pretreatment (15 min) and subsequent cooling. Endogenous peroxidase activity was

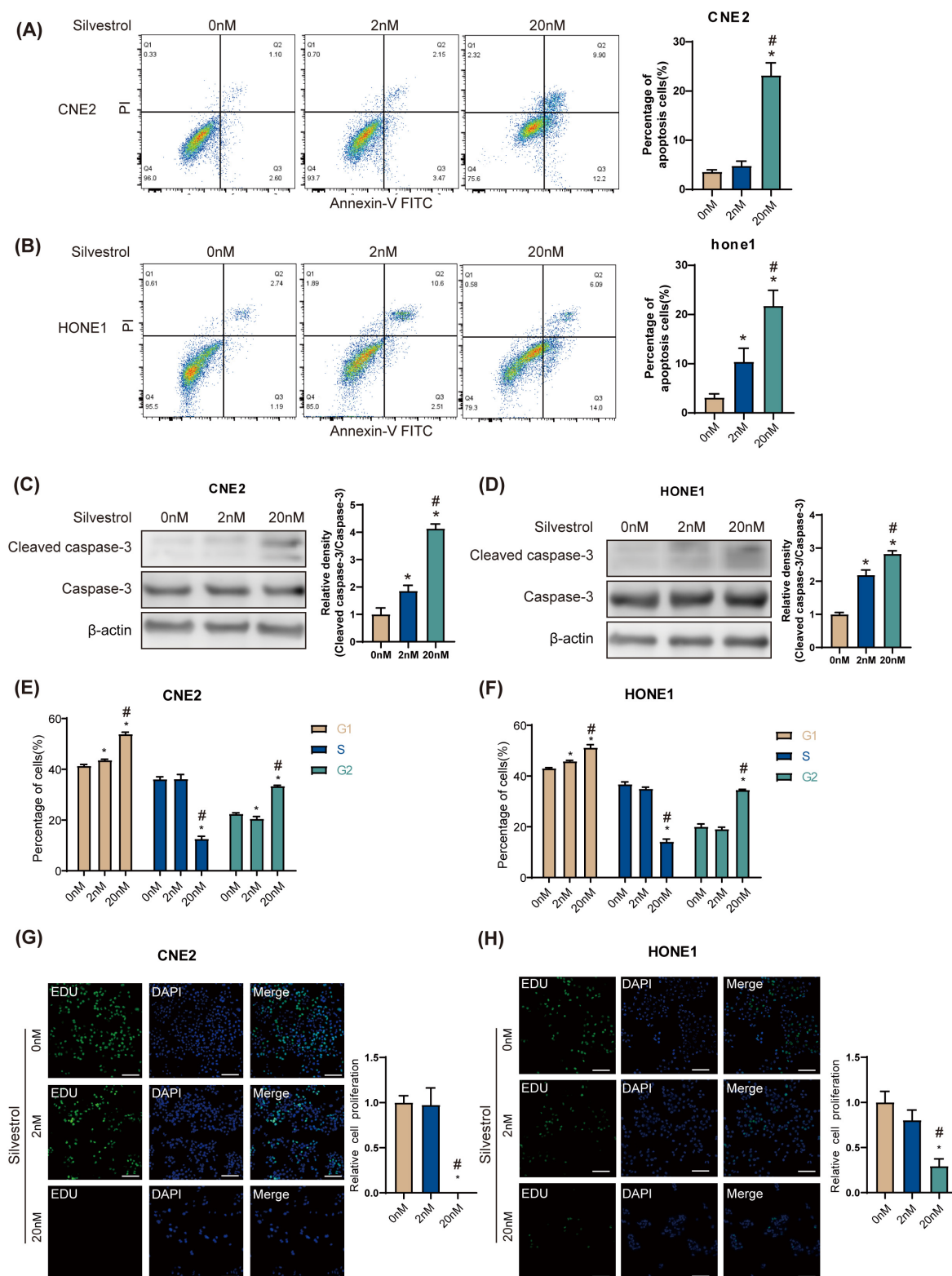


Fig. 1. Silvestrol promotes apoptosis and inhibits proliferation of nasopharyngeal carcinoma (NPC) cells. NPC cells were treated with silvestrol at different concentrations (0 nM, 2 nM, 20 nM) for 24 h. (A,B) Apoptosis was determined by flow cytometry in CNE2 and HONE1. (C,D) Western blot (WB) determined cleaved caspase-3 expression in CNE2 and HONE cells. (E,F) Cell cycle was determined by flow cytometry in CNE2 and HONE1. (G,H) Cell proliferation was measured by 5-ethynyl-2'-deoxyuridine (EdU) in CNE2 and HONE1. The values are performed as mean $\pm$ standard deviation (SD), N = 3/group. * $p < 0.05$ vs 0 nM, # $p < 0.05$ vs 2 nM. Bar = 100 μ m.

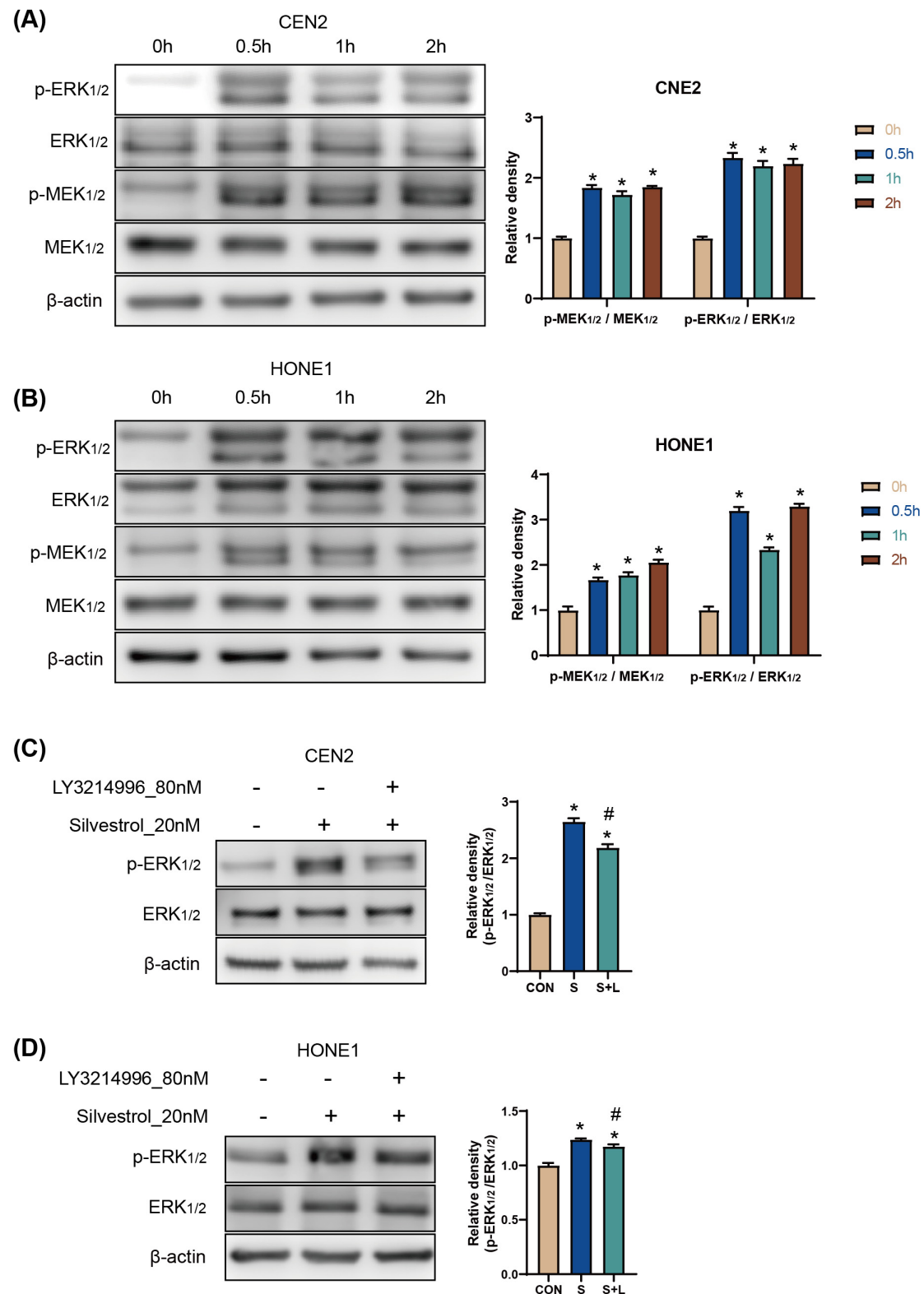


Fig. 2. Silvestrol activates the ERK pathway by promoting ERK phosphorylation. (A,B) WB detection for p-ERK/ERK, and p-MEK/MEK protein expression in CNE2 and HONE1 cells. Silvestrol for 0 h, 0.5 h, 1 h and 2 h. The values are performed as mean $\pm$ SD, $N = 3/\text{group}$, $*p < 0.05$ vs 0 h. (C,D) WB detection for p-ERK1/2 and ERK1/2 in CNE2 and HONE1 cells. NPC cells were treated with silvestrol and/or LY3214996 for 1h (CON, control group without drug; S, single-drug group with 20 nM silvestrol; S + L, two-drug group with 20 nM silvestrol and 80 nM LY3214996), The values are performed as mean $\pm$ SD, $N = 3/\text{group}$. $*p < 0.05$ vs CON, $\#p < 0.05$ vs S. ERK, extracellular signal-regulated kinase; MEK, mitogen-activated protein kinase kinase.

quenched with H₂O₂ (ZSGB, PV-9001, Beijing, China). Sections were incubated with 5% normal goat serum (Bioss, C0005, Bethesda, MA, USA) for 1 h at room temperature, followed by overnight incubation with primary antibodies (Affinity, DF6622/ DF6157/ DF6176, Wuhan, China) at 4 °C. The next day, goat anti-rabbit IgG polymer labeled with enhanced enzyme (ZSGB, PV-9001, Beijing, China) was applied and incubated at 37 °C for 30 minutes. Color development was performed using the DAB color reagent kit (ZSGB, ZLI-9018, Beijing, China), while nuclear staining was performed with a 0.1% nuclear solid red staining solution (Solarbio, G1320, Beijing, China). Sections were dehydrated, made transparent, and mounted with a coverslip.

2.8 RNA Sequencing and Data Analysis

CNE2 and HONE1 cells were separated into two experimental groups: silvestrol (20 nM), and silvestrol (20 nM) + LY3214996 (80 nM). Cell samples were frozen in TRIzol reagent (Solarbio, 15596-018, Beijing, China) and submitted to Geneplus Technology Corporation (Shenzhen, China) for RNA sequencing. Any mRNA with a fold-change $|FC-1| > 0.5$ and $p < 0.01$ was considered to be a differentially expressed gene (DEG). DEGs were further screened based on The Comparative Toxicogenomics Database (CTD) and transcriptome information using Venn diagram analysis. Hub genes showing the same expression trend in both cell lines were identified and categorized into up- or down-regulated groups. These up-regulated and down-regulated DEGs were then analyzed by Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway analysis and protein interaction network analysis using STRING Version 9.1 (<http://www.string-db.org>).

2.9 Statistical Analysis

Prism (GraphPad Software, GraphPad 8.0.1, San Diego, CA, USA) was used to compare groups. All data were expressed as the mean $\pm$ standard deviation (SD). The unpaired *t*-test was used to determine statistically significant differences between two groups. A *p*-value < 0.05 was considered to indicate a statistically significant difference.

3. Results

3.1 Silvestrol Promotes Apoptosis and Inhibits the Proliferation of NPC Cells

Flow cytometry revealed that 20 nM silvestrol significantly increased the apoptosis rate in HONE1 and CNE2 NPC cells ($*p < 0.001$) (Fig. 1A,B). The apoptosis rate of HONE1 and CNE2 cells also increased after treatment with 2 nM silvestrol, but this increase was only statistically significant for HONE1 cells ($*p = 0.025$) (Fig. 1B). Western Blot (WB) assay showed that the expression of cleaved caspase-3 protein in CNE2 and HONE1 cells increased with increasing concentrations of silvestrol. Specifically, the expression in the 2 nM silvestrol group was significantly

higher than in the control group with 0 nM silvestrol ($*p < 0.001$; Fig. 1C, 0 nM vs 2 nM, $*p = 0.005$). The expression of cleaved caspase-3 after 20 nM silvestrol treatment was significantly higher compared to 0 nM silvestrol ($*p < 0.001$) and 2 nM silvestrol ($\#p < 0.001$) (Fig. 1C,D). Treatment with 20 nM silvestrol significantly increased the proportion of G1 phase cells more than treatment with 2 nM ($\#p < 0.001$) and 0 nM silvestrol ($*p < 0.001$) (Fig. 1E,F). The proportion of cells in S phase and G2 phase after treatment with 2 nM silvestrol either decreased or remained unchanged, with only the decrease in G2 phase for CNE2 cells showing statistical significance ($*p = 0.018$) (Fig. 1E,F). The proportion of CNE2 and HONE1 cells in the G1 and G2 phases following treatment with 20 nM silvestrol was significantly higher than after treatment with 0 nM silvestrol ($*p < 0.001$) or 2 nM silvestrol ($\#p < 0.001$) (Fig. 1E,F). After treatment with 20 nM silvestrol, the proportion of CNE2 and HONE1 cells in S phase was significantly lower than with 0 nM silvestrol ($*p < 0.001$) or 2 nM silvestrol ($\#p < 0.001$) (Fig. 1E,F). The EdU assay showed that 20 nM silvestrol significantly reduced the relative cell proliferation ratio of CNE2 and HONE1 cells compared with 0 nM ($*p < 0.001$) and 2 nM silvestrol ($\#p < 0.001$). No significant difference was observed between 2 nM and 0 nM silvestrol (Fig. 1G,H). The above results indicate that silvestrol promotes apoptosis in NPC cells and inhibits their proliferation. Moreover, these effects are dose-dependent.

3.2 Silvestrol Activates the ERK Pathway by Increasing the Phosphorylation Levels of ERK and MEK

The results of the Western Blot (WB) assay showed that p-ERK and p-MEK protein levels were significantly increased in CNE2 and HONE1 cells between 0.5 h and 2 h after silvestrol treatment ($*p < 0.001$) (Fig. 2A,B). A selective inhibitor of ERK, LY3214996, reversed the silvestrol-induced phosphorylation of ERK in NPC cells. Treatment with 20 nM silvestrol significantly increased p-ERK in CNE2 and HONE1 cells compared to the control ($*p < 0.001$) (Fig. 2C,D). Treatment with 80 nM LY3214996 in combination with 20 nM silvestrol significantly reduced the level of p-ERK protein compared to treatment with silvestrol alone ($\#p < 0.001$; Fig. 2D).

3.3 Silvestrol Acts on NPC Cells through the ERK Pathway

Treatment with 20 nM silvestrol increased the apoptosis rate of HONE1 and CNE2 cells significantly ($*p < 0.001$) (Fig. 3A,B). Co-treatment with 80 nM LY3214996 (an ERK pathway inhibitor) and 20 nM silvestrol significantly reduced the proportion of apoptotic cells compared to silvestrol treatment alone ($\#p < 0.001$; Fig. 3A, $\#p = 0.007$) (Fig. 3A,B). Similarly, Western Blot (WB) results showed that 20 nM silvestrol significantly increased the expression of cleaved caspase-3 ($*p < 0.001$) (Fig. 3C,D). Treatment of CNE2 and HONE1 cells with 80 nM LY3214996 and 20 nM silvestrol significantly reduced cleaved caspase-3

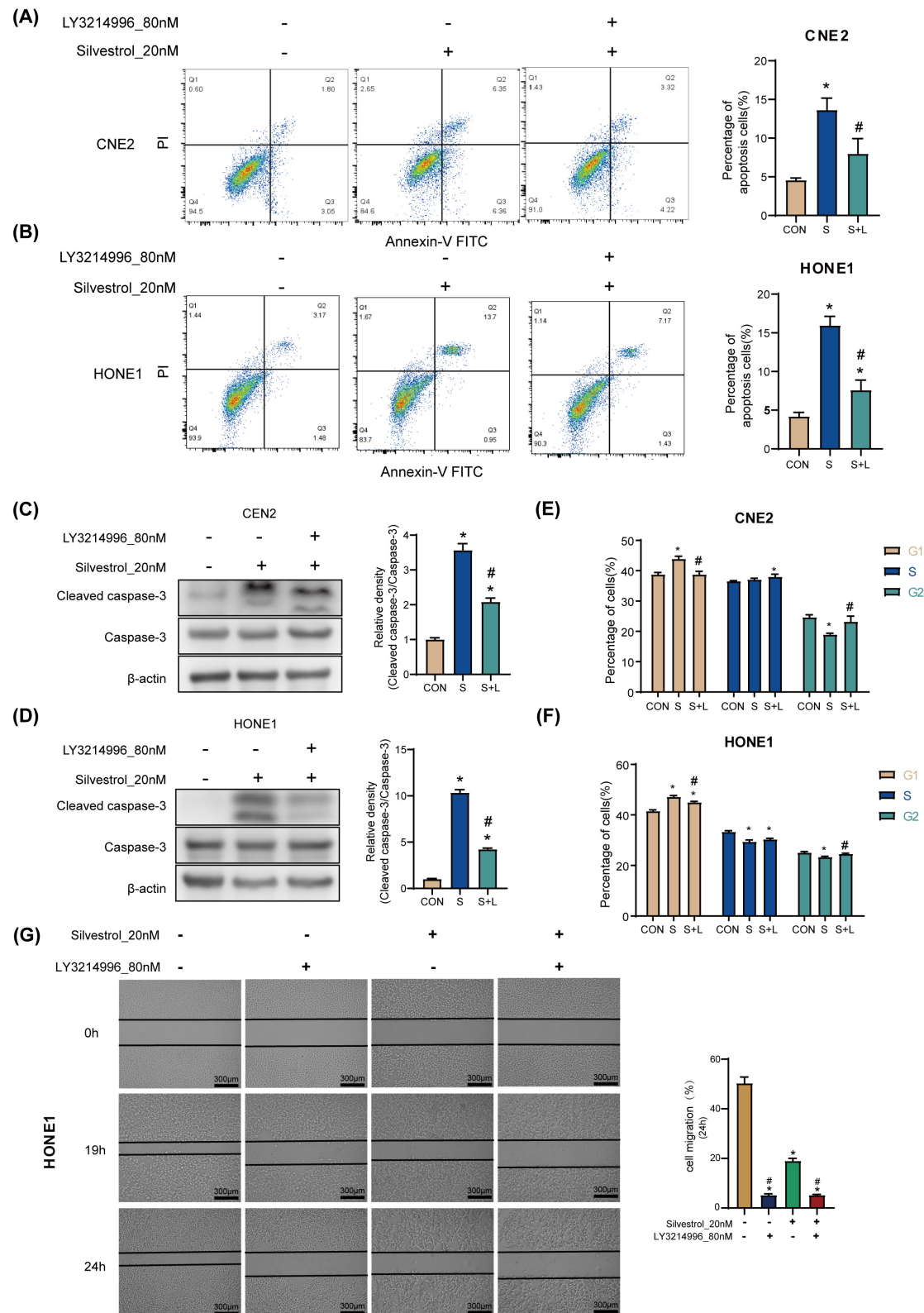


Fig. 3. Silvestrol acts on NPC cells through the ERK pathway. (A,B) Apoptosis was determined by flow cytometry in CNE2 and HONE1. (C,D) WB detection for cleaved caspase-3 expression in CNE2 and HONE cells. (E,F) Cell cycle was determined by flow cytometry in CNE2 and HONE1. (G) Cell migrations of HONE1 in response to different drug treatments. NPC cells were treated with silvestrol and/or LY3214996 for 1h (CON, control group without drug; S, single-drug group with 20 nM silvestrol; S + L, two-drug group with 20 nM silvestrol and 80 nM LY3214996), Bar = 300 μ m; The values are performed as mean $\pm$ SD, N = 3/group. * $p < 0.05$ vs CON, # $p < 0.05$ vs S.

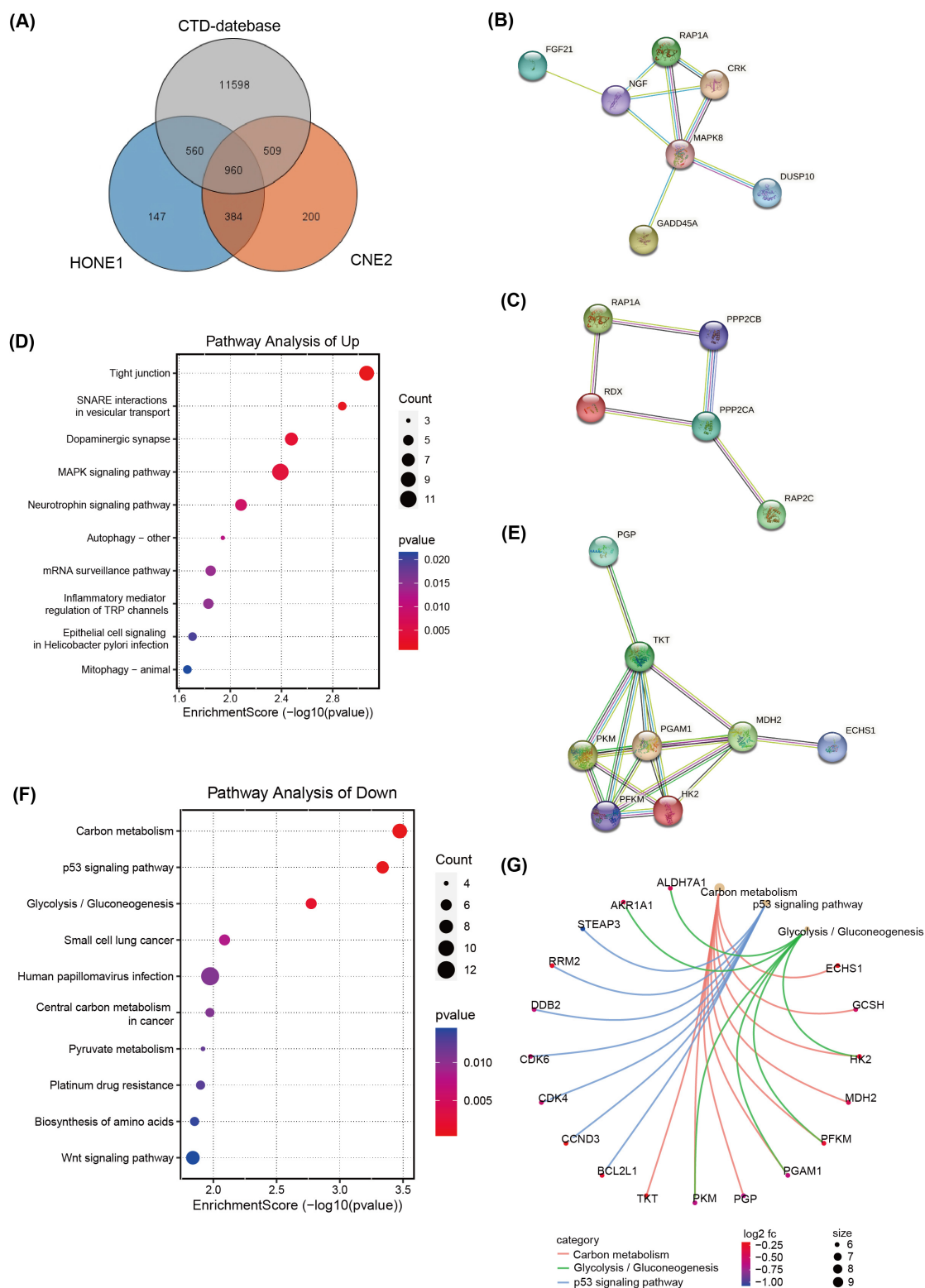


Fig. 4. Transcriptome differences in NPC cells treated with silvestrol and with or without ERK inhibitors. (A) Venn diagram of Comparative Toxicogenomics Database (CTD) database, TCGA and RNA-seq. (B,C) The protein-protein interaction (PPI) network of up-regulated hub DEGs ($|FC-1| \geq 0.5$). (D) KEGG pathway enrichment of up-regulated hub DEGs. (E) The PPI network of down-regulated hub DEGs ($|FC-1| \geq 0.5$). (F) KEGG pathway enrichment of down-regulated hub DEGs. (G) The DEG network of key pathways. TCGA, The Cancer Genome Atlas; RNA-seq, RNA sequencing; DEGs, differentially expressed genes; KEGG, Kyoto Encyclopedia of Genes and Genomes; FC, fold change.

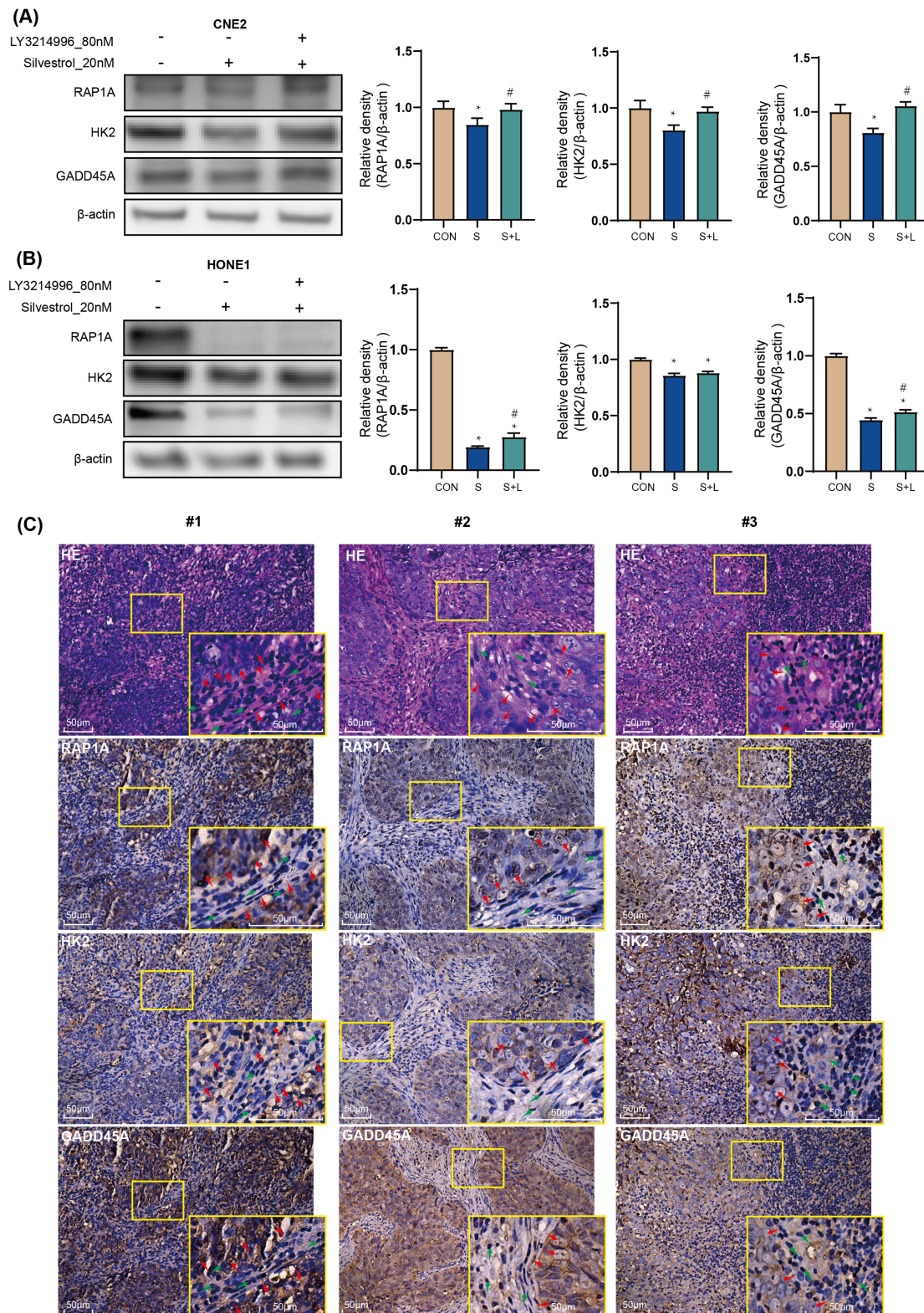


Fig. 5. Target genes: the effect of silvestrol on protein expression in NPC patient tissue. NPC cells were treated with silvestrol with or without LY3214996 for 1 h (CON, control group; S, 20 nM silvestrol; S + L, 20 nM silvestrol and 80 nM LY3214996). The expression level of GADD45A, RAP1A and HK2 proteins was evaluated by WB assay in (A) CNE2 cells, and (B) HONE1 cells. (C) H&E tissue sections from NPC patients, and IHC of GADD45A, RAP1A and HK2. Red arrows, tumor cells; Green arrow, non-tumor cells. Bar = 50 μ m. The values are performed as mean $\pm$ SD, N = 3/group. * p < 0.05 vs CON, # p < 0.05 vs S. H&E, hematoxylin & eosin; IHC, immunohistochemistry; GADD45A, growth arrest and DNA damage inducible alpha; RAP1A, Ras-related Protein 1A; HK2, hexokinase 2.

expression compared to silvestrol treatment alone ($\#p < 0.001$) (Fig. 3C,D). Flow cytometry revealed that 20 nM silvestrol significantly increased the proportion of HONE1 and CNE2 cells in the G1 phase compared with the control ($*p < 0.001$), and significantly decreased the proportion of cells in the S phase and G2 phase ($*p < 0.001$), except for CNE2 cells in the S phase, which did not show a significant decrease (Fig. 3E,F). Co-treatment with 80 nM LY3214996 and 20 nM silvestrol significantly reduced the proportion of CNE2 and HONE1 cells in the G1 phase ($*p < 0.001$) and significantly increased the proportion of G2 phase cells ($\#p < 0.001$), indicating a reversal of silvestrol's cell cycle arrest effect (Fig. 3E,F). The statement about a trend for increased S phase cells compared with silvestrol alone needs clarification; it implies that co-treatment with LY3214996 might modulate the cell cycle differently, but specific data or a statistical value should be provided for a clear understanding. In the cell migration assay, although the initial scratch width was almost the same for each group at 0 h, the group treated with LY3214996 and silvestrol showed a wider scratch width after 19 h and 24 h, indicating inhibited cell migration compared with the control and the silvestrol alone groups (Fig. 3G). This result suggests that the ERK pathway might play a role in cell migration, which silvestrol can influence, and that LY3214996 can modulate this effect.

3.4 Transcriptome Differences in NPC Cells Treated by Silvestrol with or without ERK Inhibitors Revealed Significant Findings

A total of 960 DEGs, related to both CNE2 and HONE1 cells as well as broader NPC research available in databases like the Comparative Toxicogenomics Database (CTD) (<http://ctdbase.org/>), were identified through Venn diagram analysis (Fig. 4A). Of these, 572 hub DEGs displayed consistent expression trends across CNE2 and HONE1 cells, comprising 303 down-regulated genes and 269 up-regulated genes. Protein network analysis of the up-regulated genes highlighted two protein interaction networks, with RAP1A protein identified as a core element in both (Fig. 4B,C). Furthermore, the tight junction pathway, involving RAP1A, emerged as the top-scoring pathway in the KEGG pathway analysis, indicating significant changes in pathways associated with the up-regulated DEGs (Fig. 4D). For the down-regulated genes, hexokinase-II (HK2), PFKM, PGAM1, and PKM proteins were all pivotal members of their respective protein interaction network (Fig. 4E). These proteins are integral to carbon metabolism and glycolysis/gluconeogenesis pathways (Fig. 4G), which were highlighted as the top three pathways in KEGG pathway enrichment analysis (Fig. 4F).

3.5 Expression of GADD45A, HK2, and RAP1A Proteins is Regulated by the ERK Pathway under the Influence of Silvestrol and is Closely Related to the Development of NPC

Treatment with 20 nM silvestrol significantly decreased RAP1A, HK2, and GADD45A protein expression in CNE2 and HONE1 cells compared to the control group ($*p < 0.001$; Fig. 5A: $*p_{\text{RAP1A}} = 0.032$, $*p_{\text{HK2}} = 0.008$, $*p_{\text{GADD45A}} = 0.009$) (Fig. 5A,B). Co-treatment with 80 nM LY3214996 (an ERK inhibitor) and 20 nM silvestrol significantly increased the expression of RAP1A, HK2, and GADD45A proteins compared with silvestrol treatment alone (Fig. 5A: $\#p_{\text{HK2}} = 0.016$, $\#p_{\text{GADD45A}} = 0.002$; Fig. 5B: $\#p_{\text{RAP1A}} = 0.006$, $\#p_{\text{GADD45A}} = 0.009$), although the increases in HK2 and GADD45A expression showed a non-significant upward trend in HONE1 cells (Fig. 5A,B). Immunohistochemistry (IHC) revealed that RAP1A is localized in the nucleus (Fig. 5C). In tumor sections from patients #1, #2, and #3, RAP1A-positive multinucleated and nuclear heterogeneous cells were observed (Fig. 5C). Hematoxylin and eosin (HE) staining showed no positive signal for RAP1A in non-tumor tissue, and a clear boundary was visible between the tumor and non-tumor regions (Fig. 5C). HK2 protein localization was identified in the cytoplasm (Fig. 5C). In tumor sections from patients #1, #2, and #3, the positive signal for HK2 around abnormal nuclei was stronger than that around normal nuclei (Fig. 5C). HE staining showed no positive signal for HK2 in the non-tumor tissue of patient #1, while a weak positive signal was observed in the non-tumor tissue of patients #2 and #3. GADD45A was detected in both the nucleus and cytoplasm (Fig. 5C), with positive areas showing an obvious boundary in the tumor sections of patients #1 and #2. Combined with HE staining and nuclear morphology, the highly positive area corresponded to the cancer nest tissue. No GADD45A positive signal was found in the non-tumor tissue of patient #1. In patient #2, the GADD45A positive signal in the non-tumor tissue was weaker than in the tumor tissue. The nuclei in patient #3 did not show GADD45A positive signal, and there was no clear boundary between the positive signal in the cancer and the non-tumor tissue.

4. Discussion

The specific location of NPC results in a relatively high risk of side effects from radiation therapy. This study investigated silvestrol, a novel small-molecule compound, for its potential to inhibit the occurrence and development of NPC. Silvestrol is known to target EIF4A, inhibiting the cellular translation process with some selectivity for mRNA [20,21]. Our findings demonstrated that silvestrol promotes cell apoptosis, inhibits cell cycle progression, and increases the phosphorylation of ERK and MEK. While previous studies have indicated that downregulation of the MEK/ERK pathway can inhibit NPC cell proliferation, migration, invasion [22,23], and promote apoptosis [24], it's

important to note that activation of the ERK pathway can both promote cell proliferation and apoptosis, suggesting its role as a double-edged sword [25]. In addition, the phosphorylation of ERK1/2 under the action of GBP3 can promote the proliferation of brain glioma cells [26].

RAP1A, a member of the RAS oncogene family, is implicated in various tumor cell processes including proliferation, invasion, migration, and apoptosis [27–30]. In addition, a study on long non-coding RNA suggests that RAP1A promotes the metastasis of NPC cells [31]. In our study, RAP1A emerged as a central node in protein interaction networks among up-regulated genes, particularly within the tight junction pathway. Despite the observed inhibition of RAP1A expression by silvestrol, co-treatment with the ERK pathway inhibitor LY3214996 reversed this effect. Immunohistochemistry (IHC) in NPC patient tumor tissue confirmed RAP1A overexpression, linking silvestrol-mediated ERK pathway activation to the regulation of RAP1A expression and its association with NPC cell apoptosis and proliferation.

HK2, a critical enzyme in glycolysis, was identified as a core member of the network formed by down-regulated genes. HK regulates the metabolic processes of aerobic glycolysis, autophagy, cell death, and intracellular Ca^{2+} flux during tumor development [32]. A previous study found that down-regulation of HK2 can increase the radiosensitivity of NPC [33]. Moreover, HK2 can attenuate the action of miR-9-1 in inhibiting NPC cell proliferation and glycolysis [34]. The mitochondrial localization of HK2 promotes the development of NPC [35]. Its involvement in key metabolic pathways and the observed downregulation of HK2 expression by silvestrol, counteracted by LY3214996 treatment (**Supplementary Fig. 1**), suggests a complex interplay in NPC cell metabolism that warrants further investigation. The discrepancies between WB and qPCR results for HK2 expression highlight the nuanced regulation of transcription and translation processes.

GADD45A, associated with growth arrest and DNA damage response [36,37], also showed differential expression in response to silvestrol and LY3214996. A study of melanoma cells found that the MAPK-ERK pathway regulates GADD45A, and that down-regulation of GADD45A promotes G2/M arrest and apoptosis [38]. Its role in cell cycle checkpoints and apoptosis aligns with our observations on cell cycle arrest and apoptosis induction in NPC cells treated with silvestrol. The variability in GADD45A expression across different patient tissues points to the need for further investigation into its regulation and role in NPC pathogenesis.

RAP1A, HK2, and GADD45A emerge as critical genes through which silvestrol may exert its anti-proliferative effects on NPC cells. However, the specific mechanisms underlying these effects and the potential interplay with the ERK pathway require deeper exploration. Further research into these genes and their regulation is vital

not only for elucidating silvestrol's antitumor mechanism but also for advancing our understanding of NPC carcinogenesis.

5. Conclusions

Silvestrol was found to affect the expression of RAP1A, HK2, and GADD45A proteins, with its action leading to the inhibition of NPC cell proliferation and the promotion of apoptosis. Specifically, silvestrol's effects on RAP1A, HK2, and GADD45A expression appear to be mediated through the activation of the ERK pathway. Moreover, silvestrol inhibits cell migration through mechanisms that do not directly involve the ERK pathway, potentially implicating the role of RAP1A in these non-ERK pathway-related effects. This dual mechanism of action highlights silvestrol's potential as a multifaceted therapeutic agent in the treatment of NPC, targeting both cell proliferation and migration to inhibit cancer progression.

Availability of Data and Materials

The datasets generated and analyzed during the current study are available in the SRA repository, [<https://dataview.ncbi.nlm.nih.gov/object/PRJNA946356?reviewer=a6s5rqba44sf6thtv4tpsibtm0>]. Additionally, other datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Author Contributions

LY led the methodology, validation, formal analysis, writing - original draft, and data curation. JF, JD, and WY conducted the validation process and collaborated with LY to finalize the writing of the “Materials and Methods” section, as well as ensuring the accuracy of the “Results” section in manuscript. DL, XQ, and XL provided supervision and project administration, were responsible for the overall project management and supervision, and participated in the conceptualization and design of the manuscript, as well as critical review of the key content. XH, DL, and YT were responsible for Writing - review and editing and Visualization; they conducted significant critical reviews and editing of the manuscript content. All authors read and approved the final manuscript. All authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

This study was conducted in accordance with the Declaration of Helsinki and approved by the Ethics Committee of Chongqing General Hospital (protocol code: KY S2022-073-01, date: 2022.03.11). Written informed consent was obtained from all participants before entering the study.

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Conflict of Interest

The authors declare no conflict of interest.

Supplementary Material

Supplementary material associated with this article can be found, in the online version, at <https://doi.org/10.31083/j.fbl2904160>.

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