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Targeted Inhibitory Effect of Nasopharyngeal Carcinoma Cells by Hre_2.Grp78 Chimeric Promoter Regulating Fusion Gene *TK/VP3*

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Abstract

Objective: To construct plasmids with Hre_2.Grp78 chimeric promoter regulating fusion gene *TK/VP3* and elaborate the effects of overexpressed *TK/VP3* on nasopharyngeal carcinoma cells. **Methods:** Four plasmids were constructed, including pcDNA3.1-CMV-TK/VP3, pcDNA3.1-Hre_2.TK/VP3, pcDNA3.1-Grp78.TK/VP3, and pcDNA3.1-Hre_2.Grp78.TK/VP3. The human nasopharyngeal carcinoma cell line HNE1 cells were transfected with the 4 plasmids, respectively. Cell viabilities were evaluated using 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, and apoptosis was conducted using flow cytometry analysis. The expression of TK, VP3, Grp78, and hypoxia-inducible factor 1α and apoptosis-related proteins was determined by real-time quantitative polymerase chain reaction and Western blotting. **Results:** The recombinant plasmids that could steadily overexpress TK and VP3 were successfully constructed. Expression of TK and VP3 in cells transfected with pcDNA3.1-Hre_2.TK/VP3 and pcDNA3.1-Grp78.TK/VP3 was significantly higher than pcDNA3.1-CMV-TK/VP3, and expression in cells transfected with pcDNA3.1-Hre_2.Grp78.TK/VP3 was the highest. Under glucose deprivation or hypoxia condition, Grp78 or hypoxia-inducible factor 1α was overexpressed so that expression of TK and VP3 was significantly upregulated, which could further inhibit cell proliferation and enhance cell apoptosis. **Conclusion:** We successfully constructed 4 plasmids with Hre_2.Grp78 chimeric promoter regulating fusion gene *TK/VP3,* which could significantly inhibit the proliferation as well as enhance the apoptosis of nasopharyngeal carcinoma cells under glucose deprivation or hypoxia condition.

Keywords

nasopharyngeal carcinoma, TK, VP3, plasmids construction

Abbreviations

cDNA, complementary DNA; GCV, ganciclovir; HIF-1α, hypoxia-inducible factor 1α; Hre, hypoxia-response element; HSV-TK, herpes simplex virus thymidine kinase; mRNA, messenger RNA; NPC, nasopharyngeal carcinoma; RT-qPCR, real-time quantitative polymerase chain reaction.

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Introduction

Nasopharyngeal carcinoma (NPC), a unique malignant epithelial carcinoma that mostly distributes in southern China and Southeast Asia, has an incidence estimated as 20 to 50 per 100 000.[1] Despite the development of therapeutic methods such as radiotherapy and chemotherapy, the prognosis of patients with NPC remains poor. Nasopharyngeal carcinoma is highly malignant, invasive, and metastatic,[2] and most patients with NPC are found to be at an advanced stage when diagnosed, and the recurrence rate of patients with NPC can be as high as 82%.[3] Studies show that genetic factors,[4] Epstein-Barr virus infection,[5] and environmental and dietary factors[6] are most related risk factors for tumorigenesis of NPC. However, deeper insights for NPC are still unclear.

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The chicken anemia virus *VP3* gene can produce a 13.6-kDa protein named apoptin,[7] which can induce apoptosis selectively in many kinds of tumor cells[8] such as laryngeal cancer,[9] gastric cancer,[10] and breast cancer.[11] Meanwhile, apoptin does not affect normal nontransformed human cells,[12,13] such as hematopoietic stem cells, endothelial cells, or primary fibroblasts, which make it a potential therapeutic target for cancer.

The suicide gene, herpes simplex virus thymidine kinase gene (*HSV-TK*), is considered as a tumor therapeutic target for several cancers.[14] The transfer of HSV-TK into a variety of tumor cells, including bladder cancer,[15] colorectal cancer,[16] and liver cancer,[17] exerts antitumor efficacy as a result of the activation of ganciclovir (GCV) to its cytotoxic triphosphate derivative that induces apoptosis in target cells.[18] Glucose-regulated protein-78 (Grp78), which belongs to the HSP70 protein family, is considered to play critical roles in the stress of oncogenesis.[19] Studies show that Grp78 is overexpressed in many cancers and plays a key role in tumor development and metastasis.[20,21] It is also considered that Grp78 can selectively regulate *HSV-TK* suicide gene and may be effective in inhibition of esophagogastric junction and gastric adenocarcinomas.[22] The regulation of Grp78 is associated with expression of hypoxia-inducible factor 1α (HIF-1α) in many bioprocesses.[23] A study also indicates the fusion gene, HSV1-TK&green fluorescent protein (*GFP*), is under the control of a HIF-1-inducible hypoxia-response element (Hre) in colorectal cancer cells.[24] However, no study reported the role of HSV-TK in NPC, and the relationship among TK, Grp78, and HIF-1α in cancer cells is still unclear.

In the present study, we aimed to overexpress both TK and VP3 in NPC cells by constructing recombinant plasmids to investigate the inhibition effects. And we also inserted Hre and Grp78 sequences into the promoters of plasmids to enhance the transcriptional activity for TK and VP3. This study may give a new potential direction for treatment of patients with NPC and can also provide better understanding for development of NPC.

Methods and Materials

Cell Culture

Nasopharyngeal carcinoma cell line HNE1 cells were purchased from ATCC (Manassas, Virginia). Briefly, the cells were cultured in glucose-deficient RPMI 1640 medium supplemented with 10% Gibco fetal bovine serum and 100 μg/mL penicillin–streptomycin (Sigma-Aldrich, Denver, Colorado) at 37 C and 5% CO_2. Cells were cultured to 30% to -50% confluence and then treated with 5 μmol/L prodrug GCV. For induction of hypoxia, the medium was placed in the hypoxia cabin under 5% CO_2 and 2% O_2, and cells were cultured for 0, 12, 24, and 48 hours at 37°C and 5% CO_2 followed with addition of 5 μmol/L GCV.

Construction of Plasmid and Cell Transfection

In the present study, 4 plasmids were constructed, including pcDNA3.1-CMV-TK/VP3, pcDNA3.1-Hre$_2$.TK/VP3, pcDNA3.1-Grp78.TK/VP3, and pcDNA3.1-Hre$_2$.Grp78.TK/VP3. Briefly, Grp78 promoter was cloned and inserted into pcDNA3.1 vector (Sigma-Aldrich, Poole, United Kingdom) to form pcDNA3.1-Grp78-CMV. Then, both pcDNA3.1-Grp78-CMV and recombinant plasmid containing *TK/VP3* fusion genes were digested by EcoRI/KpnI and formed pcDNA3.1-Grp78.TK/VP3-CMV. Similar procedures were performed to obtain pcDNA3.1-Hre$_2$.TK/VP3-CMV. For construction of pcDNA3.1-Hre$_2$.Grp78.TK/VP3, pcDNA3.1-Grp78.TK/VP3 was digested by NheI/XhoI, and Hre with NheI/ XhoI enzyme cutting site was used to form pcDNA3.1-Hre$_2$.Grp78.TK/VP3-CMV. Finally, CMV was moved by NruI and NheI. The sequence of the plasmids was determined using an automatic sequencer (ABI Prism 3100 Genetic-Applied Biosystems/Perking Elmer, California). Cells were transfected with corresponding vectors and control vectors using the lipo6000 reagent (Beyotime Biotechnology, China) according to the manufacturer's instruction.

Measurement of Cell Viability

Cell viabilities were evaluated using 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Briefly, cells were seeded at density of 3×10^3 in 96-well plates and cultured for 48 hours at 37°C and 5% CO_2. Then, 10-μL MTT solution (5 mg/mL) was added, and the cells were subsequently cultured for 4 hours at 37°C and 5% CO_2. After the MTT was removed, the supernatant was replaced with 200 μL dimethyl sulfoxide. The value of optical density was evaluated at 490 nm as described previously.[25]

Measurement of Apoptosis

Cell apoptosis was conducted using flow cytometry analysis. Briefly, cells were seeded into 6-well plates at density of 3×10^5/well and were stained with Annexin V/PI double staining kit (BD Biosciences, Massachusetts) strictly according to the manufacturer's instruction. Cell apoptosis was then analyzed using a FSCAN flow cytometer (BD Biosciences). All experiments were performed in triplicate.

Real-Time Quantitative Polymerase Chain Reaction

The expression of TK, VP3, Grp78, and HIF-1α was determined using real-time quantitative polymerase chain reaction (RT-qPCR). Briefly, total RNA was extracted from NPC cells by Trizol reagent (Tiangen Biotech, Beijing, China) according to the manufacturer's instructions. Then RNA was converted into complementary DNA (cDNA) using a Prime-Script 1-step RT-qPCR kit (Takara, Dalian, China). And, PCRs were performed using Green PCR Master Mix (Takara) in an ABI7500 System (Applied Biosystems, Foster City, California). Primers used in PCR were as follows:

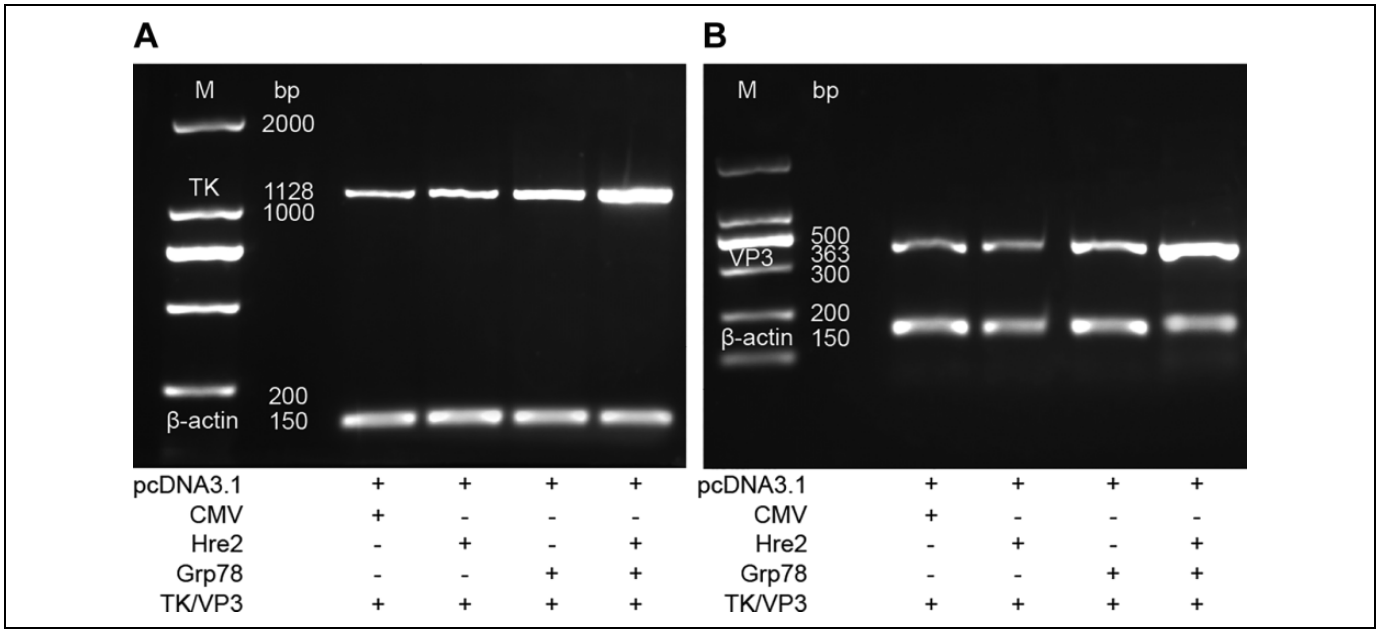

Figure 1. Recombinant plasmids expressing TK or/and VP3 were successfully constructed under normal condition. A, DNA gel electrophoresis for TK. B, DNA gel electrophoresis for VP3.

TK, F 5′-CTCGAGATGGTCGCGTCTGCGTTCGAC-3′,
R 5′-GTCGACTCAGTTAGCCTCCCCCATC-3′;
VP3, F 5′-GCTTCCATGGCATTAAAAGAGGAGATTT-TTGATCAA-3′,
R 5′-GCTTGGTACCTTACATAGAGTCATAAATTACT-GATGTGCT-3′;
Grp78, F 5′-AACACCCGAGAAAAGTGCCGAG-3′,
R 5′-AATGTGGACCAGTCAGTCGCTC-3′;
HIF-1α, F 5′-ATCCATGTGACCATGAGGAAATG-3′,
R 5′’-TCGGCTAGTTAGGGTACACTTC-3′;
β-actin, F 5′-CACTCTTCCAGCCTTCCTT-3′,
R 5′-CGGACTCGTCATACTCCT-3′.

Relative RNA levels were calculated by the $2^{-\Delta\Delta C}$ method. β-Actin was used as an internal control.

Western Blotting Analysis

The protein levels of TK, VP3, Grp78, and HIF-1α as well as p21, p53, APC1, cytochrome C, and caspase-3 were determined by Western blotting. Briefly, total proteins were extracted from NPC cells, and the proteins were quantified with protein assay reagent from Bio-Rad (Hercules, California). Proteins were then loaded on 10% sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride or polyvinylidene difluoride membranes. The membranes were subsequently blocked using 5% nonfat milk at room temperature for 1 hour. And then membranes were probed with the specific primary antibodies (all purchased from Abcam [Cambridge, MA, USA]) against TK (anti-thymidine kinase 1 antibody, ab76495, 1/5000), VP3 (anti-VP3 antibody, ab193612, 1/5000), Grp78 (anti-Grp78 antibody ab21685, 1/500), HIF-1α (anti-HIF-1α antibody, ab113642,1/500), p21 (anti-p21 antibody, ab109520, 1/1000), p53 (anti-p53 antibody, ab26, 1/500), APC1 (anti-Apc1 antibody, ab133397, 1/500), cytochrome C (anti-cytochrome C antibody, ab13575, 1/500), caspase-3 (anti-caspase-3 antibody, ab13585, 1/500) at 4°C overnight and subsequently incubated with corresponding secondary antibody at 37°C for 45 minutes. Protein bands were scanned with the Pierce ECL Western Blotting Substrate (Pierce, Shanghai, China). β-Actin was served as an internal control.

Statistical Analysis

The measurement data were expressed by mean (standard deviation). Comparisons were conducted using Student *t* test for comparison of 1 groups and 1-way analysis of variance for comparison of 3 or more groups. A P value <.05 was considered to be significantly different. All calculations were made using SPSS 20.0 (SPSS Inc, Chicago, Illinois).

Results

Recombinant Plasmids Expressing TK or/and VP3 Were Successfully Constructed Under Normal Condition

First, we constructed 4 recombinant plasmids, pcDNA3.1-CMV-TK/VP3, pcDNA3.1-Hre$_2$.TK/VP3, pcDNA3.1-Grp78.TK/VP3, and pcDNA3.1-Hre$_2$.Grp78.TK/VP3. Polymerase chain reaction results showed that TK mRNA was at 1128 bp and VP3 was at 363 bp (Figure 1A and B).

After transfection, the expression of TK and VP3 was determined by RT-qPCR and Western blotting. As shown in Figure 2, in all cells transfected with the recombinant plasmids, both TK and VP3 were detected, and the expression in cells transfected with pcDNA3.1-Hre$_2$.TK/VP3 or pcDNA3.1-

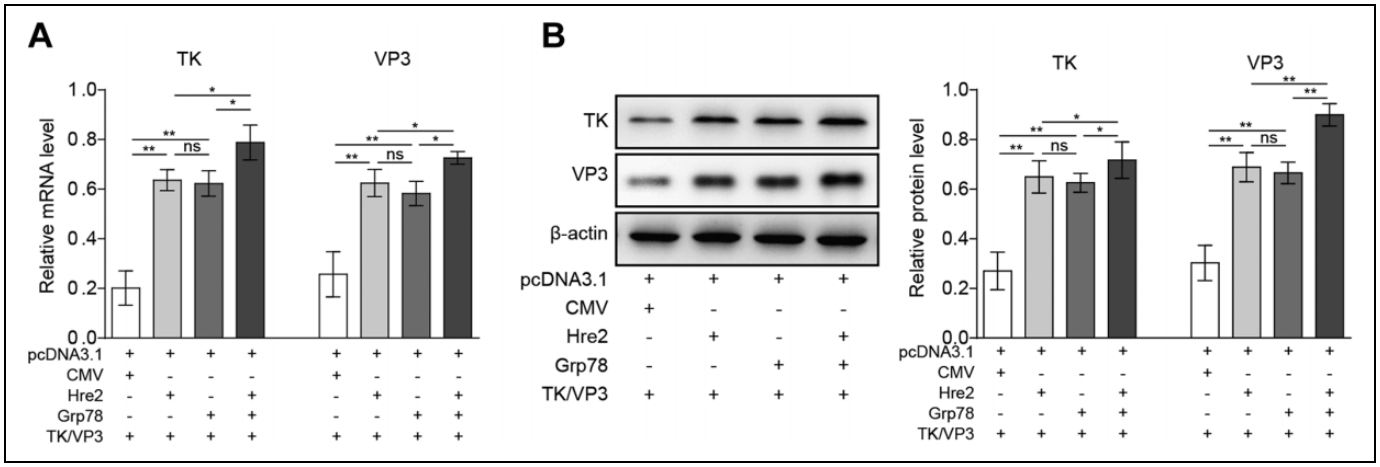

Figure 2. Recombinant plasmids expressing TK or/and VP3 were successfully constructed under normal condition. A, The mRNA expression of TK and VP3 was determined by RT-qPCR. B, The protein expression of TK and VP3 was determined by Western blotting. The mean (standard deviation) in the graph presents the relative levels from 3 replications. ns > .05,*P < .05, **P < .01. mRNA indicates messenger RNA; RT-qPCR, real-time quantitative polymerase chain reaction.

Grp78.TK/VP3 was significantly higher (almost 3.2-fold) than in cells transfected with pcDNA3.1-CMV-TK/VP3 (P < .05). Besides, expression in cells transfected with pcDNA3.1-Hre$_2$.Grp78.TK/VP3 was the highest (P < .05). These results suggested that the recombinant plasmids expressing TK or/and VP3 were successfully constructed, and the insertion of both Hre and Grp78 in the promoter could significantly enhance the transcriptional activity of both TK and VP3.

Overexpressed TK and VP3 Could Inhibit Proliferation and Enhance Apoptosis of NPC Cells Under Glucose Deprivation

To further investigate effect of overexpressed TK/VP3 on proliferation and apoptosis of NPC cells, we measured cell viability and apoptosis of cells transfected with different plasmids under glucose deprivation. Results showed that the cell proliferation significantly decreased gradually in groups with the increasing expression of TK and VP3 compared with cells transfected with the pcDNA3.1 plasmids (control group; P < .05, Figure 3A). At 48 hours, the proliferation of cells transfected with pcDNA3.1-Hre$_2$.Grp78.TK/VP3 reduced to almost 0.15-fold of control cells. Apoptosis results showed cells with higher TK and VP3 levels had higher apoptosis rates compared to the control group. The apoptosis rate of cells transfected with pcDNA3.1-Hre$_2$.Grp78.TK/VP3 was almost 4-fold of the control group cells (P < .05, Figure 3B).

The messenger RNA (mRNA) and protein expression levels of Grp78, TK and VP3 were shown separately in Figure 3C and D. Similar to the above, in all cells transfected with the 4 plasmids, the expression of TK and VP3 was significantly higher compared with control group (P < .05). In cells transfected with pcDNA3.1-Hre$_2$.Grp78.TK/VP3, the mRNA level of TK was almost 3.1-fold and VP3 was almost 4.0-fold than that of control group in Figure 3C, and similar results were observed for the protein levels of TK and VP3 in Figure 3D. Besides, the mRNA and protein expression of Grp78 was also significantly higher in cells transfected with pcDNA3.1-Grp78.TK/VP3 (2.1-fold and 3.2-fold, respectively) and pcDNA3.1-Hre$_2$.Grp78.TK/VP3 (3.3-fold and 4.7-fold, respectively), showing the construction was successful. When Grp78 was overexpressed, the expression of TK and VP3 was significantly enhanced (P < .05), indicating the increase in Grp78 might upregulate the expression of both TK and VP3 under glucose deprivation.

Overexpressed TK and VP3 Could Inhibit Cell Proliferation and Enhance Cell Apoptosis of NPC Cells Under Hypoxia Condition

To further study the effects of overexpressed TK and VP3 on NPC cells, NPC cells under hypoxia condition were also transfected with the above 4 plasmids as well as the control vectors pcDNA3.1 under hypoxia condition. When TK and VP3 were overexpressed, the cell viability was significantly decreased and apoptosis was significantly increased compared to the control cells (P < .05; Figure 4A and B). However, in cells transfected with pcDNA3.1-CMV-TK/VP3 or pcDNA3.1-Grp78.TK/VP3, the decreased proliferation was significantly inhibited than cells transfected with pcDNA3.1-Hre$_2$.TK/VP3 or pcDNA3.1-Hre$_2$.Grp78.TK/VP3 (P < .05). Meanwhile, apoptosis was obviously augmented in the cells transfected with plasmids containing Hre sequences, where the effect was more significant than that containing Grp78.

The mRNA and protein expression level of HIF-1α, Grp78, TK, and VP3 in cells with pcDNA3.1-Hre$_2$.TK/VP3 or pcDNA3.1-Hre$_2$.Grp78.TK/VP3 were significantly higher compared with control cells and cells with pcDNA3.1-CMV-TK/VP3 (Figure 4C and D). Besides, we also observed when HIF-1α was upregulated, the expression of Grp78 also increased significantly; however, overexpressing Grp78 alone

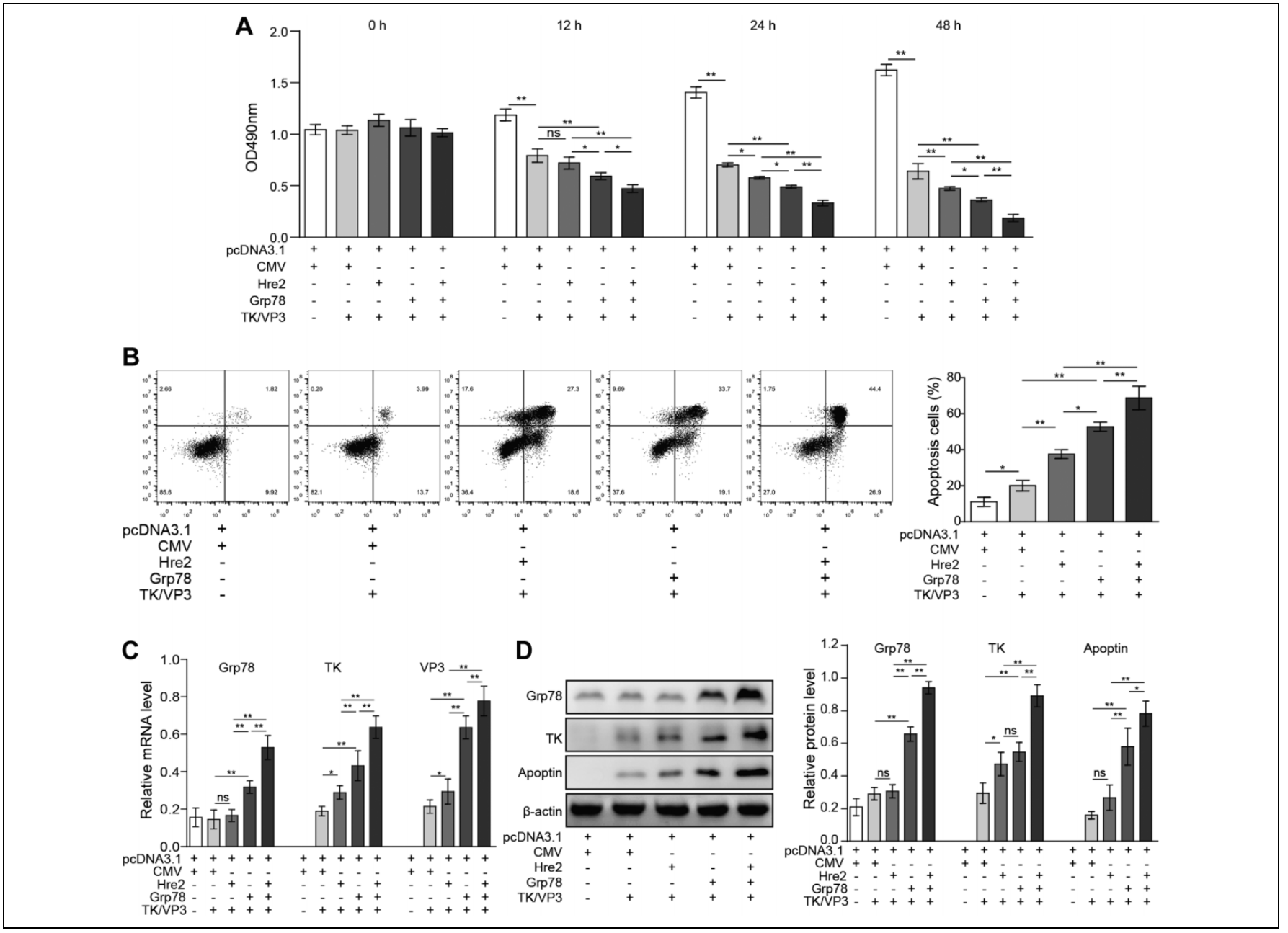

Figure 3. Overexpressed TK and VP3 suppressed proliferation and enhanced apoptosis of NPC cells under glucose deprivation. A, Cell viability for cells with different plasmids by MTT assay. B, Cell apoptosis assay for cells with different plasmids by FCM analysis. C, The mRNA expression of TK, VP3, and Grp78 for cells with different plasmids was determined by RT-qPCR. D, The protein expression of TK, VP3. and Grp78 for cells with different plasmids was determined by Western blotting. The mean (standard deviation) in the graph presents the relative levels from 3 replications. ns > .05,*P < .05, **P < .01. FCM, flow cytometry; mRNA, messenger RNA; MTT; NPC, nasopharyngeal carcinoma; RT-qPCR, real-time quantitative polymerase chain reaction.

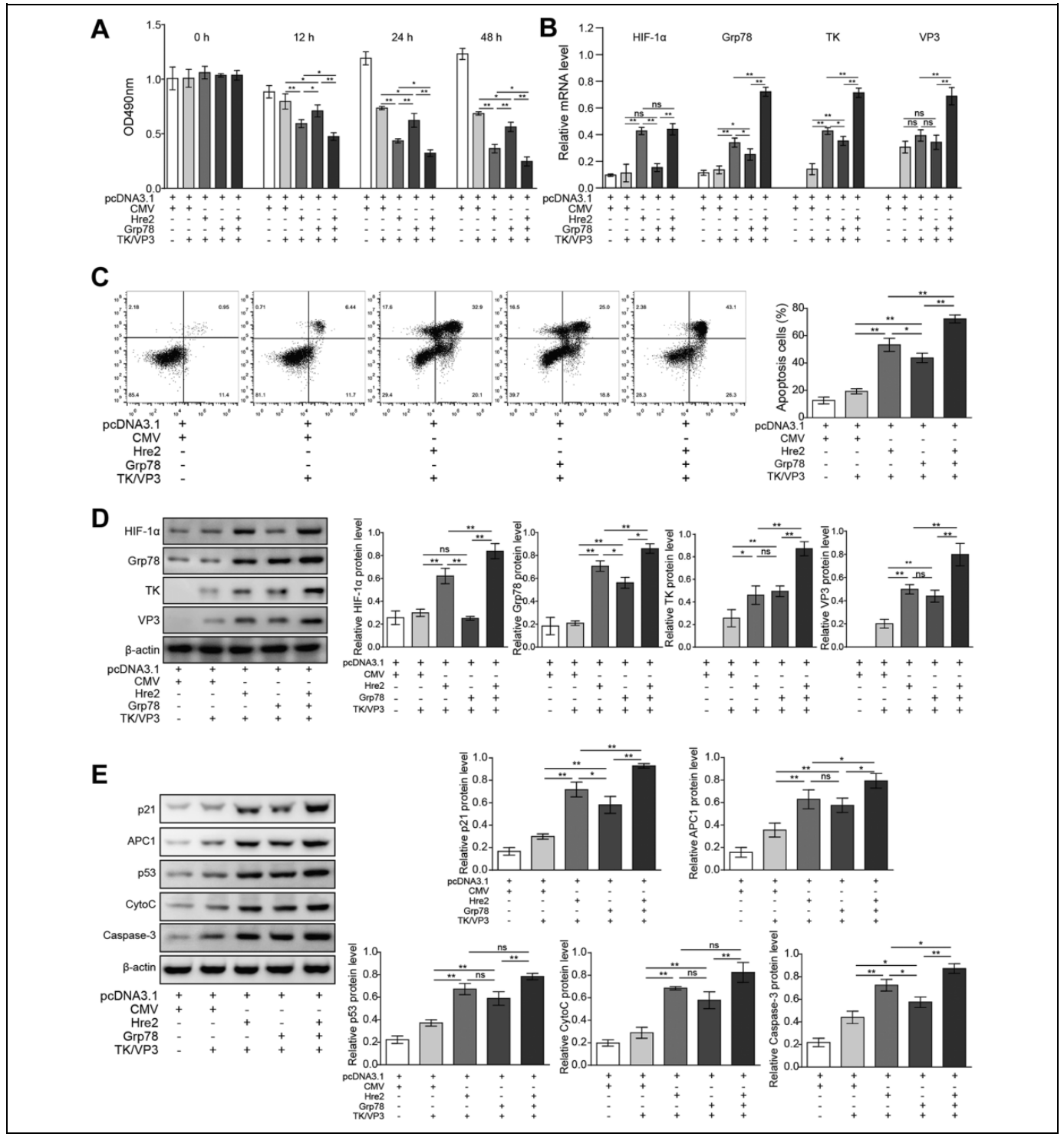

Figure 4. Overexpressed TK and VP3 could inhibit cell proliferation and enhance cell apoptosis of NPC cells under hypoxia condition. A, Cell viability under hypoxia condition by MTT assay. B, Cell apoptosis assay under hypoxia condition by FCM analysis. C, The mRNA expression of TK, VP3, Grp78, and HIF-1α under hypoxia condition was determined by RT-qPCR. D, The protein expression of TK, VP3, Grp78, and HIF-1α under hypoxia condition was determined by Western blotting. E, The protein expression of p21, p53, APC1, cytochrome C, and caspase-3 under hypoxia condition was determined by Western blotting. The mean (standard deviation) in the graph presents the relative levels from 3 replications. ns > .05, *P < .05, **P < .01. FCM, flow cytometry; HIF-1α, hypoxia-inducible factor 1α; mRNA, messenger RNA; MTT; NPC, nasopharyngeal carcinoma; RT-qPCR, real-time quantitative polymerase chain reaction.

didn't affect the expression of HIF-1α, suggesting Grp78 was regulated by HIF-1α under hypoxia condition.

Finally, the expression of apoptosis-related proteins p21, p53, APC1, cytochrome C, and caspase-3 was determined in different group of NPC cells. As shown in Figure 4E, in cells with all plasmids, all expression of the abovementioned proteins was significantly increased compared with the control ($P < .05$). And with the increase in TK and VP3 levels, the expression of the apoptosis-related proteins also gradually increased.

Discussion

Due to the highly malignant, invasive, and metastatic characteristics, NPC is considered one of the most common malignant cancer in the world .[26] Until now, the therapeutic strategies for NPC have been limited, and the prognosis of patients with NPC is still poor mainly due to mono-radiotherapy or concurrent adjuvant.[27]

It had reported that VP3 can induce apoptosis selectively in many kinds of tumor cells, including NPC. Fang *et al* found that combination of VP3 with photodynamic therapy could induce NPC cell death both *in vitro* and *in vivo*.[28] Besides, Azatian *et al* also demonstrated that Grp78 could selectively regulate *HSV-TK* suicide gene that could be used as potential therapeutic method for sophagogastric junction and gastric adenocarcinomas.[22] In the present study, we successfully constructed recombinant plasmids to express TK or/and VP3 to probe into the anticancer effects of TK or/and VP3 on NPC cells. We also inserted Hre and Grp78 sequences into the promoters of plasmids to enhance the transcriptional activity for TK or/and VP3.

When Grp78 and Hre were inserted into the promoters, one or both, the expression of TK and VP3 significantly enhanced. In a recent study, Liang *et al* investigated effect of *MPPa-PDT* and *HSV1-TK/GCV* gene therapy on prostate cancer and used Grp78 promoter to enhance the expression of HSV1-TK in prostate cancer cells.[29] Early in 2004, Dong *et al* demonstrated that the Grp78 promoter could inhibit tumors in murine cells by driving expression of the *HSV-TK* suicide gene.[30] All those were in consistent with our findings.

Hypoxia-inducible factor 1α is a transcription factor that is involved in diverse aspects of cellular and physiologic processes, including cancer development. The regulation of Grp78 is associated with expression of HIF-1α in many bioprocesses. Interestingly, we also observed that overexpressed HIF-1α could enhance the expression of Grp78.[31] Many studies have already demonstrated the regulating effect of HIF-1α on Grp78. Lee e*t al* showed that HIF-1α-Grp78-Akt signaling pathway played an important role in hypoxic preconditioning of mesenchymal stem cells.[32] Fang *et al* found plasminogen kringle 5 could suppress gastric cancer by regulating both HIF-1α and Grp78.[23] Thus, it is reasonable to deduce that expression of TK was upregulated by HIF-1α–enhanced Grp78.

Although abovementioned studies have provided additional evidences and support for our findings, to the best of our knowledge, yet no study showed relationships among VP3, Grp78, and HIF-1α. Thus, we are the first to show the expression of VP3 might be also regulated by HIF-1α/Grp78 signaling, which absolutely needs more studies to confirm.

The antitumor activity of TK and VP3 has been noticed in many studies. Li *et al* showed combination of HSV1-TK/shTERT by retrovirus vector could inhibit hepatocellular carcinoma cell growth both *in vitro* and *in vivo*.[33] Wu *et al* showed VP3 could suppresses breast cancer cell growth *in vitro* and *in vivo*.[11] Since NPC is a solid tumor containing many hypoxic cells, we studied effects of highly expressed TK and VP3 on cell proliferation and apoptosis of NPC under glucose deprivation or hypoxia conditions. Results showed that the overexpressed TK and VP3 could significantly inhibit cell proliferation as well as enhance cell apoptosis of NPC. What's more, highly expressed TK and VP3 could also inhibit expression of apoptosis-related proteins, and the process might be associated with p53/p21-induced apoptosis signaling, APC/C signaling, and caspase-3 pathway, which needs more studies to show deeper insights. The present study also has some limitations, deeper insights for how TK and VP3 influence NPC cells are still needed; the influence of TK and VP3 on cell invasion and migration should be further studied, and the results should be confirmed in more NPC cell lines.

In conclusion, we successfully constructed 4 plasmids, including Hre_2.Grp78 chimeric promoter regulating fusion gene *TK/VP3* to overexpress TK and VP3 and study the effects of highly expressed TK and VP3 on NPC cells. Results showed that the overexpression of TK and VP3 could significantly suppress the cell proliferation and enhance the cell apoptosis of NPC under glucose deprivation or hypoxia conditions. This study may give a new potential direction for the treatment of patients with NPC.

Declaration of Conflicting Interests

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