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Research Article

Effect Of Puerarin on The Growth of Nasopharyngeal Carcinoma Cells and Its Impact on Angiogenesis

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Abstract

Objective

Puerarin is a form of isoflavones obtained from Pueraria lobata. It stimulates hepatic metabolic function and lowers serum ALT, AST, and total-bilirubin level. The purpose of this study was to examine the effect of puerarin on nasopharyngeal carcinoma (NPC) CNE1 cells and preliminarily explore its possible mechanism.

Materials and Methods

CCK8 method was used to detect the proliferation activity of puerarin on NPC CNE1 cells and IC50 was calculated. CNE1 cells were treated with 0 μmol/L puerarin (containing equal volume of DMSO solution) as control group and 1000 μmol/L puerarin (IC50 concentration) as experimental group. Colony formation assay, Scratch-wound test and Transwell invasion assay were used to detect the clone formation ability, migration and invasion ability of puerarin on CNE1 cells. Then, RNA Sequencing was used to detect the changes of differentially expressed genes (DEGs) and signaling pathways after puerarin was applied to CNE1 cells.

Results

The inhibitory effect of puerarin on the proliferation activity of CNE1 cells was enhanced with the increase of concentration, and IC50 was calculated as 1000 μmol/L. Compared with the control group, the treatment of CNE1 cells with 1000 μmol/L puerarin could inhibit the clone formation, migration and invasion of CNE1 cells ($P<0.05$). A total of 379 DEGs were found by RNA sequencing, including 295 down-regulated genes and 84 up-regulated genes (padj<0.05). The significant differences in biological functions of differentially expressed genes were mainly distributed in “negative regulation of growth”, “angiogenesis”, “regulation of peptidase activity”, “positive regulation of vasculature development”, “digestion”, “positive regulation of angiogenesis”, “negative regulation of peptidase activity”, “extracellular matrix” and “Golgi lumen” (padj<0.05).

Conclusion

Puerarin could inhibit the proliferation, migration and invasion of NPC CNE1 cells, and its mechanism might be related to the inhibition of angiogenesis and cell growth.

Keywords: cne1 cells; puerarin; cell proliferation; rna sequencing

Introduction

Nasopharyngeal Carcinoma (NPC) is an epithelial cancer that occurs in the nasopharyngeal mucosa. It is one of the common malignant tumors of head and neck. It has obvious geographical distribution characteristics and high incidence in Southeast Asia and southern China [1,2]. In southern China, the incidence rate is as high as 25/100,000-50/100,000 [3], and non-keratinizing NPC accounts for more than 95%, mainly related to Epstein-barr Virus (EBV) infection [4]. Due to the particularity of NPC anatomy and radiotherapy sensitivity, intensity-modulated radiotherapy (IMRT) has become an important treatment for NPC. NPC patients are difficult to be found in the early stage, and 75 % of the patients are diagnosed in the late stage [5]. Distant metastasis in locally advanced NPC patients is the main reason for treatment failure, and the 5-year survival rate is still only 50-60% in metastatic patients [6, 7].

The role of traditional Chinese medicine in tumor treatment has become increasingly prominent. Traditional Chinese medicine can alleviate

adverse reactions such as nausea and vomiting caused by chemotherapy [8]. In addition, some traditional Chinese medicine components can play an anti-tumor role by improving the immunity of the body [9]. Flavonoids are one of the active components of traditional Chinese medicine, which can play a role in anti-tumor. Puerarin is an isoflavone compound extracted from Puerariae Lobatae Radix. Its molecular formula is $C_{21}H_{20}O_9$ and its relative molecular mass is 416, which can be dissolved in organic solvents such as dimethyl sulfoxide (DMSO). It has been reported that puerarin has the effects of protecting myocardial cells, lowering blood pressure, anti-oxidation and reducing inflammatory response [10-12]. Studies have found that puerarin can lead to cancer cell death by regulating different mechanisms, including oxidative stress, cell cycle, Phosphatidylinositol 3-Kinase/ Pmtein Kinase B (PI3K / AKT) pathway, Mitogen- activated Protein Kinases/ Extracellular Signal-regulated Kinases (MAPK/ERK) pathway, Nuclear Factor κB (NF-κB) pathway, etc. [13]. The role of puerarin in non- small cell lung cancer showed that puerarin inhibited the proliferation of NCI-H441 and NCI-H460 cells, and induced autophagy through PI3K/Akt and MAPK/Erk signaling pathways [14]. Puerarin can inhibit the proliferation and promote apoptosis of bladder cancer T24 cells, and can inactivate NF-κB signaling pathway [15]. Puerarin can also act on liver cancer [16], ovarian cancer [17], cervical cancer [18], esophageal cancer [19], and gastric cancer [20] through different mechanisms. However, there are few studies on puerarin in NPC. In this study, the effects of puerarin on the proliferation, migration and invasion of NPC CNE1 cells were observed in vitro, and RNA sequencing (RNA-seq) was used to detect the changes of differentially expressed genes and possible pathways after puerarin was applied to CNE1 cells, providing a theoretical basis for the clinical application of puerarin in NPC.

Materials and Methods

Cell Culture

Nasopharyngeal carcinoma CNE1 cells obtained from Cell Institute of Shanghai Academy of Life Sciences were cultured in RPMI Medium 1640 containing 10% fetal bovine serum in a 5% CO_2 incubator at 37°C for routine culture. 0μmol/L puerarin treated CNE1 cells as control group, and IC50 concentration of puerarin treated CNE1 cells as experimental group.

Cell proliferation assay

CCK8 assay was used to examine the proliferation of the cells. CNE1 cells in logarithmic growth phase were seeded into 96-well plates (5×10^3/well). After 12 h of cell culture, the proliferation of CNE1 cells was assessed. The medium containing 0 μmol/L, 100 μmol/L, 200 μmol/L, 500 μmol/L and 1000 μmol/L puerarin was added to each group, and the cells were cultured for 48 h. CCK8 reagent 10 μL was added to each hole, and the absorbance of each hole was measured at 450 nm. The experiment was repeated three times. Calculate Half maximal inhibitory concentration (IC50).

Colony formation assay

About 200 CNE1 cells were inoculated in each well of the 6 -well plate, cultured for 12 h, and added with the medium containing 0 μmol/L and 1000 μmol/L puerarin, respectively. After 48 hours, cells were cultured in fresh 1640 complete medium at a constant temperature of 37˚C with 5% CO_2 for 10-14 days.Then, medium were discarded, and cells were fixed with methanol for 30 minutes at room temperature, dyed with crystal violet for 15 minutes at room temperature. Under a light microscope, the number of colonies in each well (more than 50 constituent cells, 1-2 mm in diameter) was manually counted under an x10 magnification.

Scratch-wound test

Approximately 1×10^5 CNE1 cells were seeded in 6-well plates. When the cell growth density was close to 100 %, 200 μL guns were used to close the line and PBS was used to gently wash off the exfoliated cells near the scratch. The serum-free 1640 medium containing 0 μmol/L and 1000 μmol/L puerarin was added respectively. The 6-well plates were observed under the microscope, and the sampling points were found and photographed at 100 ×. At this time, the image was recorded as 0 h. The 6- well plates were put back into the cell incubator for further culture. After 24 h, 48 h and 72 h, the images were collected once each time, and the scratch area was calculated by Image J software.

Transwell invasion assay

100μl of Matrigel diluted with serum-free 1640 medium was added to the transwell chamber, and placed in a 37°C, 5% CO_2 cell culture box before solidification. The cells treated with 0 μmol/L and 1000 μmol/L puerarin were collected and resuspended with serum-free 1640 medium. 200 μL cell suspension was added into the invasive chamber, with about 1×10^5 cells in each chamber. The lower chamber was supplemented with 600 μL 1640 medium containing 10% FBS. the cells were incubated for 24 h in 37°C, 5% CO_2 incubator. The medium in the chamber was discarded, fixed with methanol at room temperature for 30 minutes, stained with crystal violet for 20 minutes, and wiped with cotton swab to remove the cells invading the chamber. Cell invasion was observed under an inverted microscope. Photographs were taken under x100, and the number of cells penetrated into each well was calculated from 10 random fields.

RNA sequencing

CNE1 cells treated with 0μmol/L and 1000μmol/L puerarin were collected. A total RNA extraction kit was used to extract RNA according to the instructions, Then, RNA degradation and contamination was monitored on 1% agarose gels. RNA purity was checked using the Nano Photometer spectrophotometer. Library preparation, Illumina sequencing, and data analysis for transcriptome sequencing were performed by Shanghai Gene Chem.

Statistical analysis

Each experiment in this study was repeated three times independently, represented by mean±sd. GraphPad Prism 9.0 software was used to count and draw pictures. The mean of the two samples (control vs treated) was compared by unpaired Student's ttest, $P<0.05$ was considered statistically significant.

Results

Puerarin inhibits the proliferation of NPC CNE1 cells.

CCK8 assay showed that puerarin decreased the proliferation activity of CNE1 cells (Figure. 1A), and IC50 was calculated as 1000 μmol/L. The colony formation assay demonstrated that puerarin reduced($P<0.05$) the colony numbers of CNE1 cells compared with the control group (Figure. 1B and 1C). These two assays confirmed that puerarin impeded the proliferation of NPC cells.

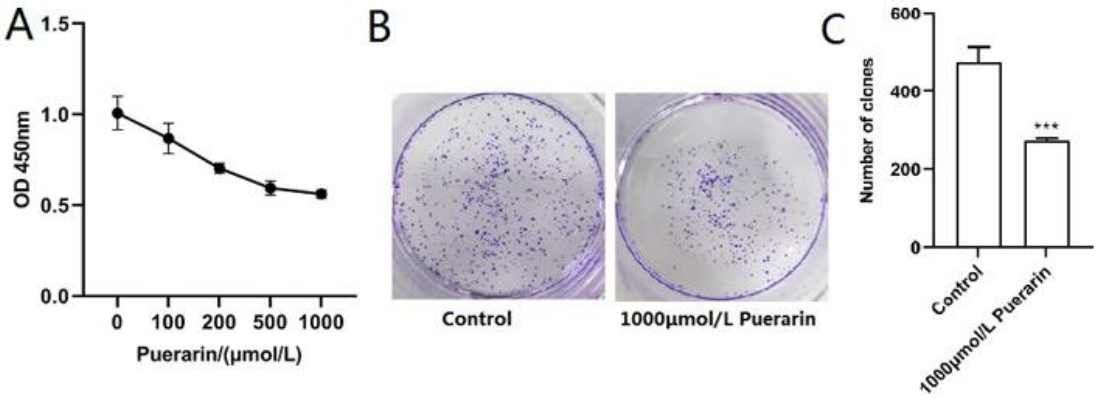

Figure 1: Puerarin inhibits proliferation of NPC CNE1 cells. **(A)** The proliferation of puerarin on CNE1 cells was detected by CCK8 assay. **(B and C)** Colony formation rate of CNE1 cells transfected with puerarin was detected using colony formation assay. Unpaired Student's t-test was used for statistical analysis. *P<0.05, **P<0.01, ***P<0.001. OD, opticaldensity.

Puerarin inhibits migration and invasion of NPC CNE1 cells

Scratch-wound test results showed that compared with the control group, 1000μmol/L puerarin group had no significant difference in healing at 24h and 48h, but decreased (P<0.05) at 72h (Fig. 2C and 2D). Transwell invasion assay demonstrated that the cell invasion of the 1000 μmol/L puerarin group was less(P<0.05) invasive, and the invasion ability of the 1000 μmol/L puerarin group was lower than that of the control group (Figure. 2A and 2B).

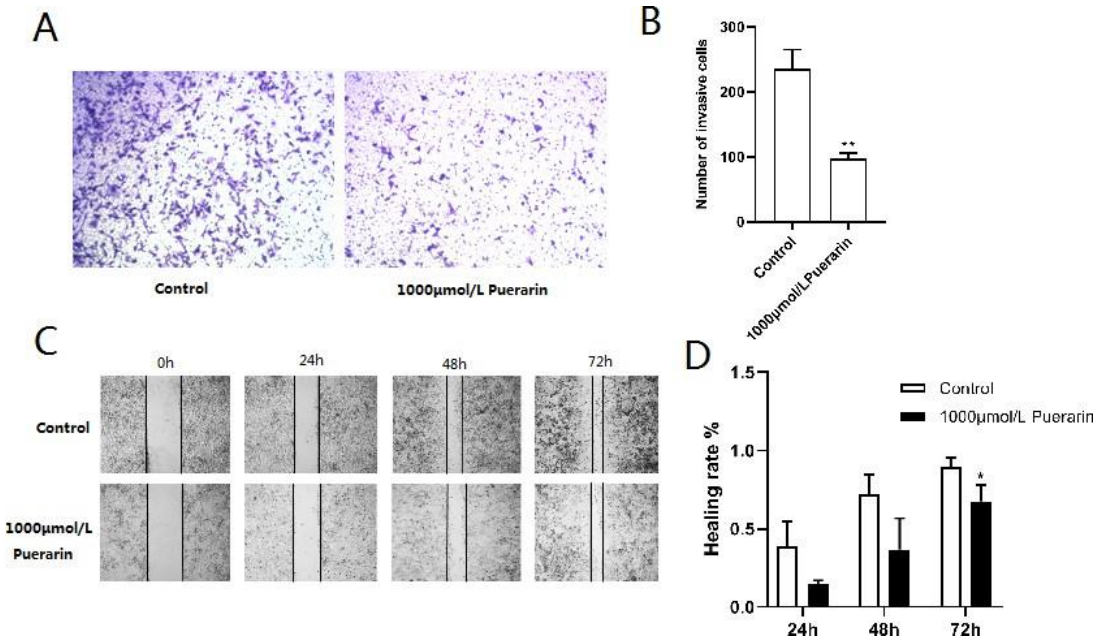

Figure 2: Puerarin inhibits migration and invasion of NPC CNE1 cells. **(A and B)** Transwell invasion assay to detect the number of invasive cells. **(C and D)** Scratch-wound test to detect cells healing rate. Unpaired Student's t-test was used forstatistical analysis. *P<0.05, **P<0.01, ***P<0.001.

Analysis of the DEGs.

The hierarchical clustering heat map of DEGs is shown in Fig. 3A. Compared with the control group, a total of 379 DEGs, 295 downregulated and 84 upregulated were detected in CNE1 cells treated with puerarin(padj<0.05) (Figs. 3B and 3C). Among the DEGs, the top 20 were annotated (Table I).

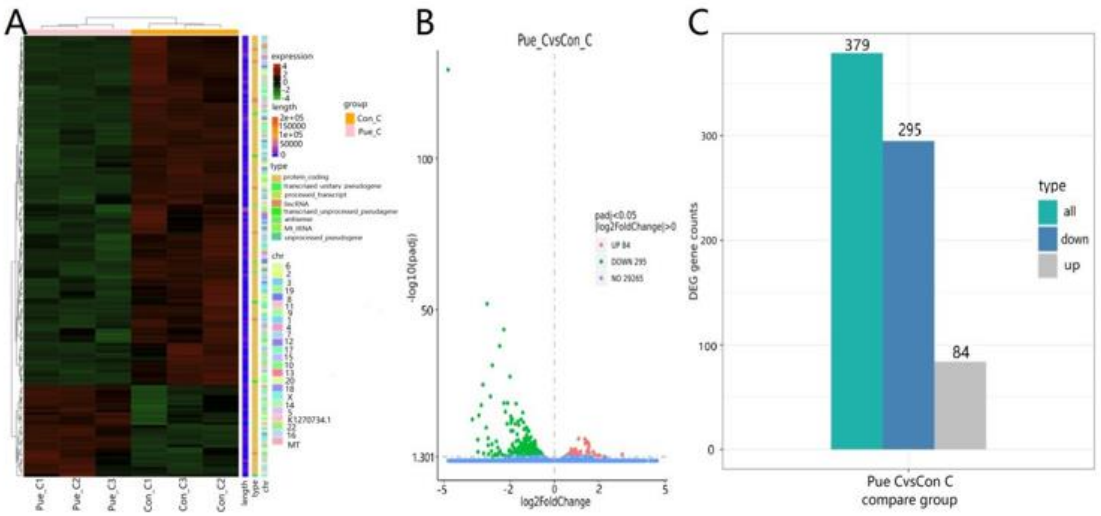

Figure 3: Analysis of the DEGs. **(A)** Heatmap of hierarchical clustering for DEGs. Red scale represented upregulated DEGs and green scale was for downregulated DEGs. **(B)** Volcano plot of DEGs. Red dots indicated upregulated DEGs and greendots indicated downregulated DEGs. **(C)** Statistical histogram of DEGs number.

ID	Padj	Name	Strand	Biotype
ENSG00000204616	$6.23x10^{-130}$	TRIM31	-	protein_coding
ENSG00000198758	$1.36x10^{-52}$	EPS8L3	-	protein_coding
ENSG00000127831	$4.58x10^{-44}$	VIL1	+	protein_coding
ENSG00000173702	$1.09x10^{-38}$	MUC13	-	protein_coding
ENSG00000155465	$3.02x10^{-32}$	SLC7A7	-	protein_coding
ENSG00000163295	$1.60x10^{-28}$	ALPI	+	protein_coding
ENSG00000060566	$7.08x10^{-26}$	CREB3L3	+	protein_coding
ENSG00000137491	$5.53x10^{-22}$	$SLCO_2B1$	+	protein_coding
ENSG00000101204	$8.72x10^{-20}$	CHRNA4	-	protein_coding
ENSG00000266714	$8.72x10^{-20}$	MYO15B	+	transcribed_unitary_pseudogene
ENSG00000168405	$1.46x10^{-19}$	CMAHP	-	protein_coding
ENSG00000187288	$4.37x10^{-19}$	CIDEC	-	protein_coding
ENSG00000101076	$6.54x10^{-17}$	HNF4A	+	processed_transcript
ENSG00000254166	$1.22x10^{-16}$	CASC19	-	lincRNA
ENSG00000255774	$9.79x10^{-16}$	AP00043.2	-	protein_coding
ENSG00000173237	$2.20x10^{-15}$	C11orf86	+	protein_coding
ENSG00000176092	$2.23x10^{-14}$	CRYBG2	-	protein_coding
ENSG00000108576	$2.23x10^{-14}$	SLC6A4	-	protein_coding
ENSG00000180316	$2.23x10^{-14}$	PNPLA1	+	protein_coding
ENSG00000107159	$2.43x10^{-14}$	CA9	+	

Table I: Top 20 significantly DEGs between control and 1000μmol/L puerarin group.

Enrichment Analysis of DEGs.

GO enrichment analysis was performed on DEGs of control group and puerarin group (Figure. 4A). It indicates that DEGs are mainly enriched in BP, such as "negative regulation of growth", "angiogenesis", "regulation of peptidase activity", "positive regulation of vasculature development", "digestion", "positive regulation of angiogenesis", "negative regulation of peptidase activity". The top 7 entries of BP were annotated (Table II).

About CC, DEGs enriched in "extracellular matrix" and "Golgi lumen". KEGG analysis showed DEGs enriched in "Complement and coagulation cascades" (padj<0.05) (Figure.4B)

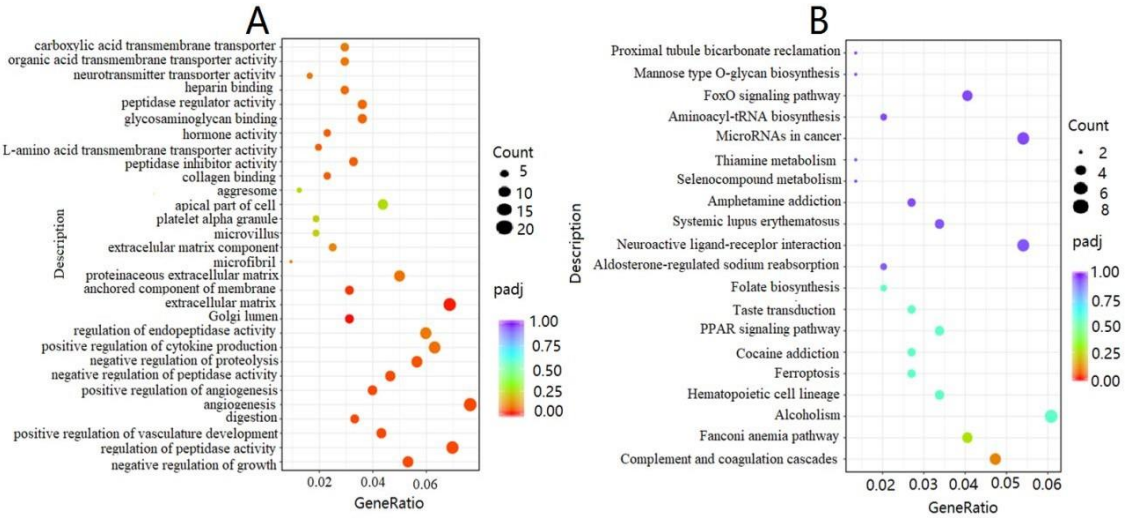

Figure 4: GO and KEGG Analysis of DEGs. **(A)** GO analysis and of the DEGs. **(B)**KEGG pathway analysis of the DEGs. GO, Gene Ontology; KEGG, Kyoto Encyclopedia of Genes and Genomes.

Category	Description	padj	GeneName	Count	Up	Down
BP	negative regulation of growth	0.033	HNF4A/SLC6A4/SERPINE2/TRIM40/SEMA5A/BCL6/PPARG/HSPA1A/HSPA1B/ALOX15B/IGFBP5/STC2/PLAC8/MT2A/WNT11/NOV	16	5	11
BP	regulation of peptidase activity	0.033	VIL1/SERPINE2/FN1/C3/F3/AQP1/SERPINE1/SSPO/CD44/MAP3K5/PPARG/SPOCK1/SERPINB1/HGF/HIP1/TFPI/PI16/TP63/LXN/IFI6/PSMB9	21	3	18
BP	positive regulation of vasculature development	0.033	C3/F3/AQP1/CTH/SERPINE1/SEMA5A/BRCA1/SRPX2/HK2/XBP1/HGF/ADM2/PLK2	13	1	12
BP	digestion	0.033	VIL1/MUC13/ALPI/GCNT3/SGK1/AQP1/AKR1C1/CAPN8/INAVA/ADM2	10	0	10
BP	angiogenesis	0.033	FN1/C3/F3/AQP1/SERPINE1/SEMA5A/BRCA1/E2F2/PPARG/SRPX2/TGFBI/HK2/XBP1/HGF/KLF5/CSPG4/ADM2/RNF213/NRP2/PARVA/NOV/ADGRG1/PLK2	23	3	20
BP	positive regulation of angiogenesis	0.033	C3/F3/AQP1/SERPINE1/SEMA5A/BRCA1/SRPX2/HK2/XBP1/HGF/ADM2/PLK2	12	1	11
BP	negative regulation of peptidase activity	0.033	VIL1/SERPINE2/C3/AQP1/SERPINE1/SSPO/CD44/SPOCK1/SERPINB1/HGF/TFPI/PI16/LXN/IFI6	14	2	12

Table 2: Top 7 entries of BP in GO enrichment analysis of DEGs.

Discussion

The results showed that puerarin could inhibit the proliferation, migration

and invasion of CNE1 cells. Studies have found that when puerarin acts on bladder cancer T24 and EJ cells, puerarin can block the G0/G1 phase cell cycle and reduce the proliferation activity of bladder cancer cells [21]. Puerarin can also inhibit the proliferation of chronic myeloid leukemia K562 cells and promote apoptosis by inducing autophagy in K562 cells [22]. Uncontrolled cell proliferation is the basic feature of cancer. The main mechanism is that the disorder of cell cycle leads to excessive proliferation and less apoptosis. Whether puerarin can inhibit the proliferation of CNE1 cells by blocking cell cycle remains unknown.Migration and invasion of tumor cells play an important role in tumor metastasis [23]. Studies have shown that puerarin can inhibit the migration and invasion of lipopolysaccharide--stimulated breast cancer cells by acting on the NF-κB pathway and Erk phosphorylation [24]. Puerarin can also inhibit the invasion and migration of ovarian cancer HO-8910 cells [25]. The incidence of NPC lymph node metastasis is high, and the cervical lymph node metastasis is found to be 70%-80% or more. Distant metastasis remains the leading cause of death in NPC patients [26]. Therefore, effective inhibition of tumor metastasis is an important part of treatment. Can puerarin control NPC metastasis by inhibiting cell migration and invasion? And the mechanism by which puerarin inhibits the migration and invasion of CNE1 cells needs further verification.

It was found by RNA-seq that the effect of puerarin on NPC may be related to the inhibition of angiogenesis and the inhibition of proliferation. It is well known that the growth of tumors requires blood vessels to provide nutrition, and blood vessels provide pathways for tumor metastasis and invasion. Vascular dysplasia exists in various types of tumors [27]. Excessive angiogenesis promotes the rapid growth of tumors, which is one of the main causes of tumor death [28]. Hypoxia is the main driver of tumor angiogenesis [29]. Radiotherapy, chemotherapy and immunotherapy can reduce the efficacy of hypoxia [30-32]. Angiogenesis and the production of vascular endothelial growth factor (VEGF) can also promote tumor growth, leading to increased expression of oncogenes or loss of tumor suppressor genes [33]. VEGF induces the expression of matrix metalloproteinases (MMPs) in NPC, which not only participates in the formation of new blood vessels by degrading the extracellular matrix of endothelial cells, but also regulates the invasion and metastasis of cancer, leading to the progress of NPC [34]. In addition, VEGF was overexpressed in nearly 70% of EBV-positive NPC patients and was associated with lymph node metastasis, recurrence and overall survival [35]. Studies have shown that Chinese herbal medicine can reduce the expression level of VEGF by inhibiting the expression of Hypoxia-inducible factor-1α (HIF-1α), and ultimately inhibit tumor angiogenesis [36,37]. NPC01 is an ancient Chinese herbal medicine prescription modified by Liang Gesang. NPC01 has the effect of inhibiting the growth of NPC cells. By inhibiting the PI3K/Akt signaling pathway, NPC01 reduces the expression of angiogenesis-related factors, including HIF-1α and VEGF [38]. Puerarin is also a kind of Chinese herbal medicine. This study found that puerarin acting on CNE1 cells may be related to inhibiting angiogenesis. Therefore, whether puerarin can play a role in NPC by inhibiting angiogenesis deserves further study.

Current cancer research efforts focus on epigenetic alterations that may be related to Sirtuin (SIRT) 1-7 [39]. In recent years, SIRT1 is considered to be the most characteristic sirtuin in seven members. And SIRT1 can be widely involved in cell processes, such as cell division, autophagy and senescence [40]. SIRT1 plays an important role in aging, metabolism and cancer [41]. SIRT1 is related to tumor cell proliferation, migration, invasion and angiogenesis [42,43]. A study found that after puerarin treatment of ovarian cancer cells, the expression of SIRT1 decreased and inhibited Wnt/β-catenin signaling pathway, thereby increasing the apoptosis of platinum-resistant ovarian cancer cells [44]. At present, many SIRT1 inhibitors have been reported, and whether puerarin can be used as a SIRT1 inhibitor related to the inhibition of NPC cells remains to be studied.

Conclusion

In summary, the results of this study showed that puerarin could inhibit the proliferation, migration and invasion of CNE1 cells in vitro, which might be related to the inhibition of angiogenesis and cell growth. The inhibitory mechanism of puerarin on NPC still needs further verification. In addition, in vivo investigation is highly recommended. This study provides a theoretical basis for the study of puerarin in nasopharyngeal carcinoma.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

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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