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Calycosin inhibits nasopharyngeal carcinoma cells by influencing EWSAT1 expression to regulate the TRAF6-related pathways

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ABSTRACT

The incidence of nasopharyngeal carcinoma (NPC) in China is relatively higher than that throughout the rest of the world, and NPC is geographically distributed. Long non-coding RNA (lncRNA) plays a key role in the development of tumors. Recent studies have found that the lncRNA Ewing sarcoma-associated transcript 1 (EWSAT1) is highly expressed in various tumors and also in NPCs. The isoflavone calycosin, which is a typical Chinese herbal medicine, can inhibit the growth of breast cancer, colorectal cancer, osteosarcoma and other cancers. The aim of our study was to select NPCs that were sensitive to calycosin and whether calycosin had an effect on NPC cells. If it does, are the effects related to a high expression of EWSAT1? We also verified that EWSAT1 was highly expressed in NPC cells. At the same time, we found that calycosin inhibited the growth of NPC cell lines. To further determine whether the effect of calycosin on NPC cells was related to EWSAT1, we used NPC cells with different concentrations of calycosin and found that the expression of EWSAT1 decreased significantly with increasing concentrations of calycosin and that the expression of downstream factors and pathways were also affected. It was demonstrated that calycosin affected NPC cell growth by regulating EWSAT1 and its downstream pathway. In addition, we overexpressed EWSAT1 and found that the increased expression of EWSAT1 weakened the growth inhibitory effect of calycosin on NPC cells.

1. Introduction

Nasopharyngeal carcinoma (NPC) is one of the most common malignant tumors in China and is the predominant malignancy of the head and neck. In China, NPC primarily occurs in Guangdong, Guangxi, Fujian and other cities in southwest China [1,2]. According to the relevant statistics, the incidence of NPC is 2–3 times higher in males than in females [3]. NPC typically occurs at a younger age in females than in males; however, the rise in incidence is similar in both sexes. The incidence of NPC has been reported to be lower at 39 years of age or younger; however, after 40 years of age, incidence increases with age and reaches a peak in the 75-year-old group [4]. In endemic areas of China, the incidence of NPC reaches 20/100000 [5–7]. Southeast Asia also has a high incidence of nasopharyngeal cancer [3]. NPC is a malignant tumor that occurs at the top and side of the nasal cavity. The causes of NPC include genetic factors, EB virus infection, environmental factors and others. Currently, the preferred method for treating NPC is radiation therapy [8], but this is the primary treatment modality for early-stage patients; the degree of control of patients with advanced NPC is unsatisfactory [9], and treatment of NPC is still a difficult problem that needs to be solved.

Phytoestrogens are divided into four main categories: isoflavones, stilbene, lignans and mushrooms [10,11]. Isoflavones are the primary phytoestrogens used in research because they are the largest biologically active components of soybeans and exhibit a significant inhibitory effect on a variety of malignant tumors [12]. Calycosin ($C_{16}H_{12}O_5$), the main isoflavone used in traditional Chinese herbal medicine, is the main component extractable from the herb Radix astragali; calycosin has been shown to exert anti-carcinogenic activities against breast cancer, osteosarcoma (OS), hepatocellular carcinoma and colorectal cancer [13–17]. However, it is unknown whether calycosin has any effect on NPCs, and if so, its specific mechanism remains unknown.

Recent research on Ewing sarcoma has shown the functionality of the lncRNA Ewing sarcoma-associated transcript 1 (EWSAT1), which is located on chromosome 15 between 2 protein-coding genes [18]. It has been reported that suppression of proliferation of Ewing sarcoma cells can be achieved via silencing EWSAT1 expression [19], which illustrates the important role that EWSAT1 plays in tumor occurrence and development [20]. At the same time, Lishan Sun et al. demonstrated through the study of EWSAT1 in OS cells that EWSAT1 can promote the

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proliferation of OS cells [19]. Recent studies have also reported that EWSAT1 is highly expressed in NPC [20], but the role it plays in NPC cells is unknown. Its specific effect and mechanism are not yet clear.

TRAF6 (tumor necrosis factor receptor- (TNFR)-associated factor 6) mediates a wide array of protein-protein interactions via its TRAF domain and a RING finger domain that contains non-conventional E3 ligase activity [21]. The TRAF6 protein in mammals is highly conserved and consists of an N-terminal Zn RING finger domain, a series of five Zn finger domains, a coiled-coil TRAF-N domain and a C-terminal TRAF-C domain [22,23]. Many studies have shown that TRAF6 is important in development [24–26], homeostasis [27] and cancer [28,29]. Therefore, we suspect that TRAF6 may also play a role in the development of NPC. It is unknown whether the expression of EWSAT1 and TRAF6 changes with the effect of calycosin on NPC cells and if there is a relationship between EWSAT1 and TRAF6.

We investigated the inhibitory effect of calycosin on NPC cells and detected the expression of EWSAT1 in NPC cells. At the same time, it was demonstrated that calycosin acted on NPC cells through EWSAT1 and its related downstream factors and pathways.

2. Materials and methods

2.1. Cell culture

The NPC cell lines CNE1, CNE2, and C666-1, as well as the normal nasopharyngeal cell line NP69, were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). The tissue source of CNE1 and CNE2 cells is nasal epithelial cells. C666-1 is a human EBV-positive nasopharyngeal carcinoma cell line. NP69 is a human immortalized nasopharyngeal epithelial cell line. CNE1, CNE2and C666-1 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, Gibco-BRL, Grand Island, NY, USA) supplemented with 10% fetal calf serum (FBS, HyClone Co. Ltd, USA). NP69 cells were cultured in keratinocyte-SFM medium (Invitrogen Corporation, USA) with the addition of 100 UmL^{-1} penicillin and 100 $\mu g\,mL^{-1}$ streptomycin. All of the cell lines were grown in a humidified incubator with 5% CO_2 at 37 °C. The cells (60% to 70% confluent) were treated with calycosin (98% purity), which was purchased from Sigma-Aldrich Ltd. (St. Louis, MO, USA) dissolved in dimethyl sulfoxide (DMSO); cells treated with vehicle (DMSO) served as the control. In some experiments, cells were pretreated for 20 h with the pCDNA3.1-EWSAT1 plasmid constructs (100 nM; XuanC Bio, Nanning, Guangxi, China) prior to treatment with calycosin.

2.2. CCK-8 assay

Calycosin was dissolved in dimethyl sulfoxide (DMSO) and stored at 4 °C for further use. The effects of calycosin on cell proliferation were measured using the CCK8 assay. CNE1, CNE2, C666-1 and NP69 cells were plated into 96-well plates (4×10^3 cells per well). Then, following an overnight incubation, the cells were treated with various concentrations of calycosin (1, 2, 4, 8, 16, 32 or 50 μM). Control cells (0 μM) were treated with 200 μL of DMEM with 10% FBS. After 12 h, 24 h or 48 h, 20 μL of CCK8 solution ($5\,g\,L^{-1}$; Sigma) in phosphate-buffered saline (PBS) was added to each well and the plates were incubated for 4 h. Finally, using a microplate reader (Bio-Tek Instruments, Winooski, VT, USA), the optical density (OD) was measured at 490 nm.

2.3. BrdU assay

Cells were treated with calycosin (50 μM) for 12 h, 24 h or 48 h. Then, the BrdU assay was performed using a BrdU Cell Proliferation Chemiluminescent Assay Kit (Cell Signaling Technology, Inc., Denver, CO, USA) according to the manufacturer's instructions.

2.4. Quantitative real-time PCR assay (qPCR)

First, RNA was extracted from CNE1, CNE2, C666-1 and NP69 cells using TRIzol (Gibco-BRL). A total of 10 ng of RNA was used for reverse transcription, which was carried out using the Revert Aid First Strand cDNA Synthesis Kit (Fermentas, Life Sciences, USA). Quantification of EWSAT1, TRAF6 and β-actin in the four cell types was performed via qRT-PCR. The primers used for RT-RCR are listed as follows: EWSAT1-F: GTGTCTGGCAAGGAACACTA, EWSAT1-R: GGTGGAGAAGAGGGAC AATAAG; TRAF6-F: TGCCATGAAAAGATGCAGAGG; TRAF6-R: GCCTG GGCCAACATTCTC; β-actin-F: CCTGGCACCCAGCACAAT; β-actin-R: GCTGATCCACATCTGCTGGAA. Then, CNE1 and CNE2 cells were treated with calycosin (0, 10, 25 and 50 μM). After 48 h, RNA was extracted and reverse transcribed into cDNA. Expression of EWSAT1 and TRAF6 in CNE1 and CNE2 cells treated with various concentration of calycosin was quantified by qRT-PCR, and GAPDH was used as a housekeeping gene to calculate the relative expression levels of EWSAT1 and TRAF6.

2.5. Western blot analysis

CNE1 and CNE2 cells were treated with calycosin (0, 10, 50 and 50 μM + EWSAT1). After 48 h, cellular lysates were harvested and the protein concentrations were determined using a Bio-Rad assay kit (Bio-Rad Laboratories, Hercules, CA, USA). Equal amounts of protein were separated by SDS–PAGE and transferred onto 0.22 μm polyvinylidene difluoride (PVDF) membranes (Bio-Rad Laboratories, USA). Then, the membranes were blocked in TBS-T (Tris-buffered saline, pH 7.6, 0.05% Tween 20) containing 5% non-fat dried milk for 2 h. The membranes were then incubated with the following primary antibodies at the indicated concentrations overnight at 4°C: TRAF6 1:1000 (Abcam, Cambridge, MA, USA), β-actin 1:1000 (Abcam, Cambridge, MA, USA), p-TAK1 1:5000 (Santa Cruz, Dallas, Texas, USA), TAK1 1:5000 (Abcam, Cambridge, MA, USA), p-IκBα 1:3000 (Santa Cruz, Dallas, Texas, USA), IκBα 1:5000 (Santa Cruz, Dallas, Texas, USA), p-c-Jun 1:5000 (Santa Cruz, Dallas, Texas, USA) and c-Jun 1:5000 (Santa Cruz, Dallas, Texas, USA). After three washes with TBST, the blots were incubated with the appropriate secondary antibodies coupled to horseradish peroxidase at room temperature for 1 h and developed in an electrochemiluminescent Western blot detection reagent (Beyotime, Jiangsu, China). Band intensities were quantified using Image-Pro Plus 5.02 software (Media Cybernetics, Bethesda, MD, USA). The expression levels of the proteins were compared with those of the control based on the relative intensities of the bands.

2.6. Mouse xenograft tumor model

The Animal Care and Use Committee of Guangxi Medical University approved the animal handling and experimental procedures. Each mouse received a subcutaneous injection of 0.2 mL of a CNE1 cell suspension (1×10^7/mL) in the right side of the axilla. Ten days after tumor cell inoculation, mice were randomly divided into three groups (10 mice per group). Mice began to receive either a DMSO (control) or calycosin (60 mg/kg or 60 mg/kg + pCDNA3.1-EWSAT1) treatment by a daily intraperitoneal injection for 28 days. Tumor volume (V) was measured every four days and calculated according to the following formula: V (mm^3) = $ab^2/2$, where a is the largest superficial diameter and b is the smallest superficial diameter. All mice were sacrificed 38 days after inoculation, and the transplanted tumors were excised and weighed.

2.7. Statistical analysis

Statistical Package for Social Sciences (SPSS) 13.0 software (SPSS, Chicago, IL, USA) was used for statistical analyses, including one-way analysis of variance (ANOVA) and Student's t-test. The data are