Bioinformatic and experimental findings to indicate anti-cancer targets and mechanisms of calycosin against nasopharyngeal carcinoma

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Research

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Abstract

Background: Calycosin is a naturally-occurring phytoestrogen that reportedly exerts anti- nasopharyngeal carcinoma (NPC) effects. Nevertheless, the molecular mechanisms for anti-NPC using calycosin remain unrevealed.

Methods: Thus, a network pharmacology was used to uncover anti-NPC pharmacological targets and mechanisms of calycosin. Additionally, validated experiments were conducted to validate the bioinformatic findings of calycosin for treating NPC.

Results: As results, bioinformatic assays showed that the predictive pharmacological targets of calycosin against NPC were TP53, MAPK14, CASP8, MAPK3, CASP3, RIPK1, JUN, ESR1, respectively. And the top 20 biological processes and pharmacological mechanisms of calycosin against NPC were identified accordingly. In clinical data, NPC samples showed positive expression of MAPK14, reduced TP53, CASP8 expressions. In studies in vitro and in vivo, calycosin-dosed NPC cells resulted in reduced cell proliferation, promoted cell apoptosis. In TUNEL staining, calycosin exhibited elevated apoptotic cell number. And immunostaining assays resulted in increased TP53, CASP8 positive cells, and reduced MAPK14 expressions in calycosin-dosed NPC cells and tumor-bearing nude mice.

Conclusion: Altogether, these bioinformatic findings reveal optimal pharmacological targets and mechanisms of calycosin against NPC, following with representative identification of human and preclinical experiments. Notably, some of original biotargets may be potentially used to treat NPC.

Background

NPC is a deadly tumor that occurs in epithelial cells, marked by high recurrence and metastasis in late-stage condition [1]. In clinical practice, NPC-induced death rate in South China is increasing recently due to the inconspicuous symptoms presenting in early stage [2]. As a result, medical inspection of NPC is basically diagnosed with advanced condition in most of NPC patients [3]. Clinically, current anti-NPC therapies are reportedly insufficient in China, such as pharmacotherapy. Further, most existing anti-NPC drugs are found with adverse side-effects following the time- and dose-dependent manners [4]. Together with present literature analysis, the proofs suggest that development of alterative anti-NPC compound is urgently needed. Traditionally, naturally-originating Chinese medicine may be a promising strategy for screening and developing bioactive components to manage diseases, including cardiovascular disorders, diabetes, cancer [5]. More notably, Astragalus root is commonly prepared as an injection for managing cardiovascular disease, inducing hepatoprotection, and adjuvant therapy of neoplasm [6]. Calycosin, rich in Astragalus root, is evidenced with beneficial activities of antioxidation, immunoregulation, cytoprotection [7]. In addition, calycosin is experimentally detected with potential pharmacological effects for suppressing cancer cells, such as osteosarcoma, hepatic carcinoma, breast cancer [8–9]. Nevertheless, the all-out pharmacological mechanism of calycosin treating NPC has not been completely uncovered. Attractively, emerging network pharmacological approach is a promising tool to reveal key biotargets and biological mechanisms of compound treating disease [10–11]. Therefore, the bioinformatic findings may be further to elucidate pharmacological targets, biological functions and signaling pathways of calycosin against NPC. Further, these systematical findings would be partially validated by conducting human and preclinical studies. Accordingly, a diagrammatic sketch from network pharmacology was displayed in visible Fig. 1.

Methods

Methods

Collection of screening targets of calycosin and NPC

Firstly, the effective tools of SwissTargetPrediction, SuperPred was employed for collecting therapeutic targets, and then NPC-associated targets were harvested by using a disease-screening database of DisGeNET. Accordingly, the identifiable targets of calycosin and NPC were further re-assayed for screening of pharmacological targets of calycosin against NPC [12].

A protein-protein interaction (PPI) map and core targets of calycosin against NPC

The tool-identified biotargets were merged to draft a connective net of calycosin against NPC by using a STRING database. And then the collective biotargets data were employed to draft a PPI map of calycosin against NPC through using a Cytoscape software. Following network settings of topological parameter, core therapeutic targets of calycosin against NPC were identified through a FunRich software [13].

Identification of molecular functions and pathways of calycosin against NPC

In brief, all therapeutic targets of calycosin against NPC were uploaded into a web-based Database of Functional Annotation Bioinformatics Microarray Analysis (DAVID) to produce optimal functional processes and signalling pathways of calycosin against NPC. Based on the settings of the -Log p-value, a three-dimensional bar chart of biological processes and signalling pathways of calycosin against NPC was created [14] .

Clinical sample and study

Briefly, 20 local patients were recruited from Department of Otolaryngology Head and Neck Surgery, the affiliated Hospital of Guilin Medical University after being medically diagnosed with NPC by using biochemical tests, clinical images, histopathological assays. The blood samples from NPC were applied for biochemical test of clinical indexes. Meanwhile, NPC samples were prepared as 5 μm sections for further specific stains. Notably, this human study was approved by the Hospital Ethics Committee, and the experimentative protocols were conducted following all terms of the Declaration of Helsinki [15–16].

A study in vitro

A human NPC cell line of CNE1 was commercially obtained from Scientific Experimental Center of Guilin Medical University (Guilin, China). And then the cells were maintained with complete growth medium of Dulbecco's Modified Eagle Medium (DMEM)/10% fetal bovine serum/1% penicillin + streptomycin within a 37 °C, 5% CO_2 incubator. After subculture for several passages, CNE1 cells were treated with different doses of calycosin (0, 20, 40, 80 μM) for 48 hours, followed with conducting the CCK8 test, flow cytometry and immunostaining assays [17].

Tumor-bearing nude mice study

Male nude mice (7-week old, specific pathogen free) were given abacterially with a density of 1×10^7 CNE1 cells via subcutaneous injection. After the tumor grown to 0.3 cm^3, the mice were dosed with calycosin (0, 20, 30, 60 mg/kg per day) for 14 days, and tumor growth was recorded every 2 days. At the end of experiment, the tumors of all mice were collected for further immunostaining tests [18].

Immunostaining steps

In brief, the samples of dewaxed human NPC, nude mice sections and fixative-fixed CNE1 cells were blocked with ready-to-use 5% bovine serum albumin buffer (Solarbio, Beijing, China) for about 1 hour. After wash with phosphate buffer saline/0.5% tween 20 buffer for at least 5 times, the samples were incubated with dilutive primary antibodies of TP53 (1:100, Bioss, Beijing, China), CASP8 (1:100, Bioss, Beijing, China), MAPK14 (1:100, Bioss, Beijing, China) at 4 °C overnight. Subsequently, the samples were further incubated with corresponding secondary antibody of IgG H&L (Alexa Fluor-488) (1:200, Abcam, USA). Intracellular nuclei were stained using a 4,6-diamidino-2-phenylindole (DAPI), and the targeting positive cells were screened and imaged by a fluorescence microscope (Olympus, Japan) before data assays [19–20].
Statistical analysis

All statistical data were presented as the means ± standard deviations. Comparisons of control and dosed groups were assessed by using a one-way analysis of variance matching following Tukey’s post hoc test. And the statistical results were conducted using a software of Statistical Product and Service Solutions 19.0 (SPSS, Chicago, IL, USA). A statistical significance concluded from a p-value less than 0.05 [21].

Results

Biological targets and PPI map of calycosin and NPC

As result, a total of 784 NPC-associated genes were identified predominantly. In addition, identified 178 genes of calycosin were harvested prior to further screening of 37 interrelated co-targets of calycosin and NPC. And then these 37 targets were used to further plot a optimal PPI map of calycosin and NPC (Fig. 2).

Analysis of PPI network parameters and selection of core targets

All mapped intersective targets were imported into Cytoscape software for identifying the topological parameters of calycosin against NPC targets and function-related PPI network. And visual analysis was conducted and shown in Fig. 3. By calculation, the median degree of freedom of the targets was 4.5, and the maximum degree of freedom was 21, and thus the core target screening condition range was set from 9 to 21. Accordingly, 8 core targets were obtained as TP53, CASP8, MAPK3, RIPK1, JUN, CASP3, MAPK14, ESR1 (Fig. 4).

Assay of biological functions and pathway enrichments of core targets

All relevant KEGG pathways of the core targets were obtained through the DAVID database and Cytoscape software to draw the core target-related pathway interaction map, as shown in Fig. 5. These top KEGG pathways were related to TNF signaling pathway, Toll-like receptor signaling pathway, Hepatitis B□Apoptosis, Viral carcinogenesis, Proteoglycans in cancer, Colorectal cancer, Pertussis, MAPK signaling pathway, Chagas disease (American trypanosomiasis), Toxoplasmosis, Epstein-Barr virus infection, Neurotrophin signaling pathway, Hepatitis C, Pathways in cancer, Tuberculosis, Herpes simplex infection, Amyotrophic lateral sclerosis (ALS), NOD-like receptor signaling pathway, RIG-I-like receptor signaling pathway. Further, relevant biological functions of the core targets were obtained through the DAVID database. The results showed that the biological processes of the core targets were mainly involved in negative regulation of I-kappaB kinase/NF-kappaB signaling,

apoptotic process, positive regulation of transcription from RNA polymerase II promoter, execution phase of apoptosis, positive regulation of macrophage differentiation, response to muscle stretch, transcription, DNA-templated, positive regulation of reactive oxygen species metabolic process, cellular response to ionizing radiation, negative regulation of transcription from RNA polymerase II promoter, Ras protein signal transduction, positive regulation of fibroblast proliferation, positive regulation of sequence-specific DNA binding transcription factor activity, angiogenesis, regulation of apoptotic process, positive regulation of I-kappaB kinase/NF-kappaB signaling, negative regulation of apoptotic process (Fig. 6).

Clinical features of patients with NPC

In an attempt to indicate some of representative targets, human NPC samples were used to conduct immunostaining tests. Following by pathological examination (HE stains) and medical scans, the NPC sections showed reduced TP53, CASP3 expressions, and elevated MAPK14 expression, in which these changed positive cells showed statistical significance compared to those in non-NPC samples ($P < 0.05$) (Fig. 7).

Pharmacological effects of calycosin against NPC cells

Further, in order to characterize the anti-NPC effects of calycosin, the studies in vitro and in vivo were assessed respectively. Interestingly, calycosin-treated NPC cells resulted in reduced cell growth ($P < 0.05$), and triggered cell apoptosis ($P < 0.05$) in a dose-dependent way. And calycosin-treated tumor-bearing nude mice showed decreased tumor mass dose-dependently ($P < 0.05$). As revealed in immunofluorescence staining, up-regulated expressions of TP53, CASP8 and reduced MAPK14 expression in calycosin-dosed NPC cells and nude mice were detected dose-dependently, in which the positive cells in calycosin groups showed statistical significance in comparison with those in controls ($P < 0.05$) (Fig. 8–9).

Discussion

NPC refers to a malignant tumor derived from epithelial tissue with fatal potentiality [22]. Clinically, the available therapeutic medicines for treating NPC are reportedly deficient, especially in advanced patients [23]. Interestingly, a functional phytoestrogen of calycosin is found with potent anti-cancer activities, such as liver cancer, breast cancer, colorectal cancer [24]. Recently, an attractive study in vitro shows calycosin inhibits proliferation of NPC cells through affecting lncRNA ewing sarcoma-associated transcript 1 (EWSAT1) expression [25]. However, the current evidence has not been revealed for detailed molecular mechanisms of calycosin against NPC. In this study, a method of network pharmacology was used to uncover the main functional procresses, and mechanisms of calycosin against NPC, as highlighted in Fig. 5–6. Taken together, it is suggested that calycosin may exert its anti-NPC effect by regulating these optimal biological processes and signaling pathways. In addition, key pharmacological biotargets of calycosin against NPC were identified respectively, as presented in Fig. 4. Therefore, some of top biotargets were representatively validated in human and preclinical samples. TP53, a well-known tumor suppressor, is reportedly studyed in many cancer topics. The molecular mechanism of anti-tumor by TP53 is involved in regulating cell growth, survival, DNA stability, and cytoprotection [26]. Pathologically, abnormal mutation of TP53 is found with many cancer human and cell samples, marked by malignant proliferation, uncontrolled growth, and strong invasiveness [27]. CASP8, a cell-death protease, autocatalytically activate initiator caspases for inducing apoptosis by proapoptotic signals. In cancer cells, CASP8 is suppressed functionally for uncontrolled proliferation, invasiveness and metastasis [28]. MAPK14, acted as an integration point for multiple biochemical signals, is implicated in a wide variety of cellular processes, including cell proliferation, differentiation, transcription regulation and development [29]. And MAPK14 is activated by various environmental stresses and proinflammatory cytokines. Overexpression of MAPK14 is one of driving etiologies of malignant cancers [30]. Collectively, the bioinformatic biotargets will be validated partially in studies in vivo and in vitro. And the human data exhibited positive expression of MAPK14, down-regulated TP53, CASP8 expressions. In addition, these experimental data showed calycosin treatments resulted in elevations of TP53, CASP8 expressions, reduction of MAPK14 expression, followed by suppressed cell proliferation, induced cell apoptosis, reduced tumor weights. As results, these findings revealed anti-proliferative effects of calycosin against NPC cells were beneficial for regulating cellular TP53, CASP8, MAPK14 expressions. Notably, the bioinformatic findings using network pharmacology might be validated in this report.

Conclusion

Taken together, emerging network pharmacology can be used to reveal therapeutic targets, functional processes and molecular mechanisms of calycosin treating NPC, marked by experimental validation. Interstingly, some of identified core targets may be potentiality for screening NPC.

Abbreviations

NPC, nasopharyngeal carcinoma; TP53, tumour protein p53; MAPK14, mitogen-activated protein kinase 14; CASP8, caspase 8; MAPK3, CASP3, RIPK1, receptor interacting protein kinase 1; TUNEL, terminal deoxynucleotidyl transferase; PPI, protein-protein interaction; DAVID, database of functional annotation bioinformatics microarray analysis; SPSS, statistical product and service solutions.

Declarations

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Availability of data and materials

All data generated or analyzed during the current study are included in this published article.

Authors' contributions

Fangxian Liu and Wenjun Huang designed and performed the experiments. Qijin Pan, Liangliang Wang and Shijiang Yi, Peng Liu analyzed the data. Fangxian Liu and Qijin Pan contributed to clinical interpretation. Fangxian Liu and Wenjun Huang drafted the manuscript. All authors have approved the final version of the manuscript and are accountable for all aspects of this study.

Ethics approval and consent to participate

Not Applicable.

Consent for publication

Not Applicable.

Competing interests

The authors declare no conflicts of interest and are responsible for the contents of this study.

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Figures

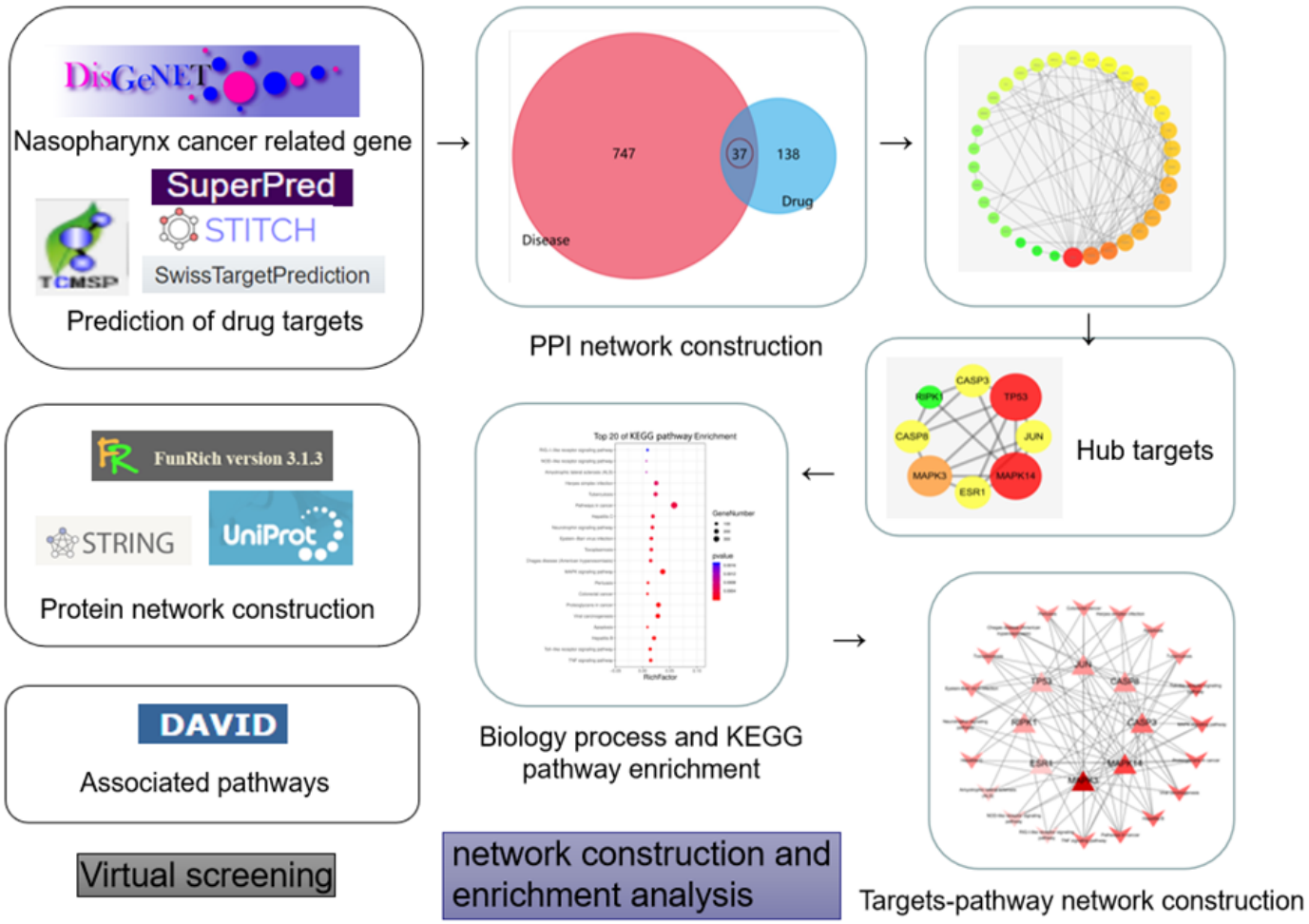

Figure 2

A network pharmacology-based diagram for the present manuscript.

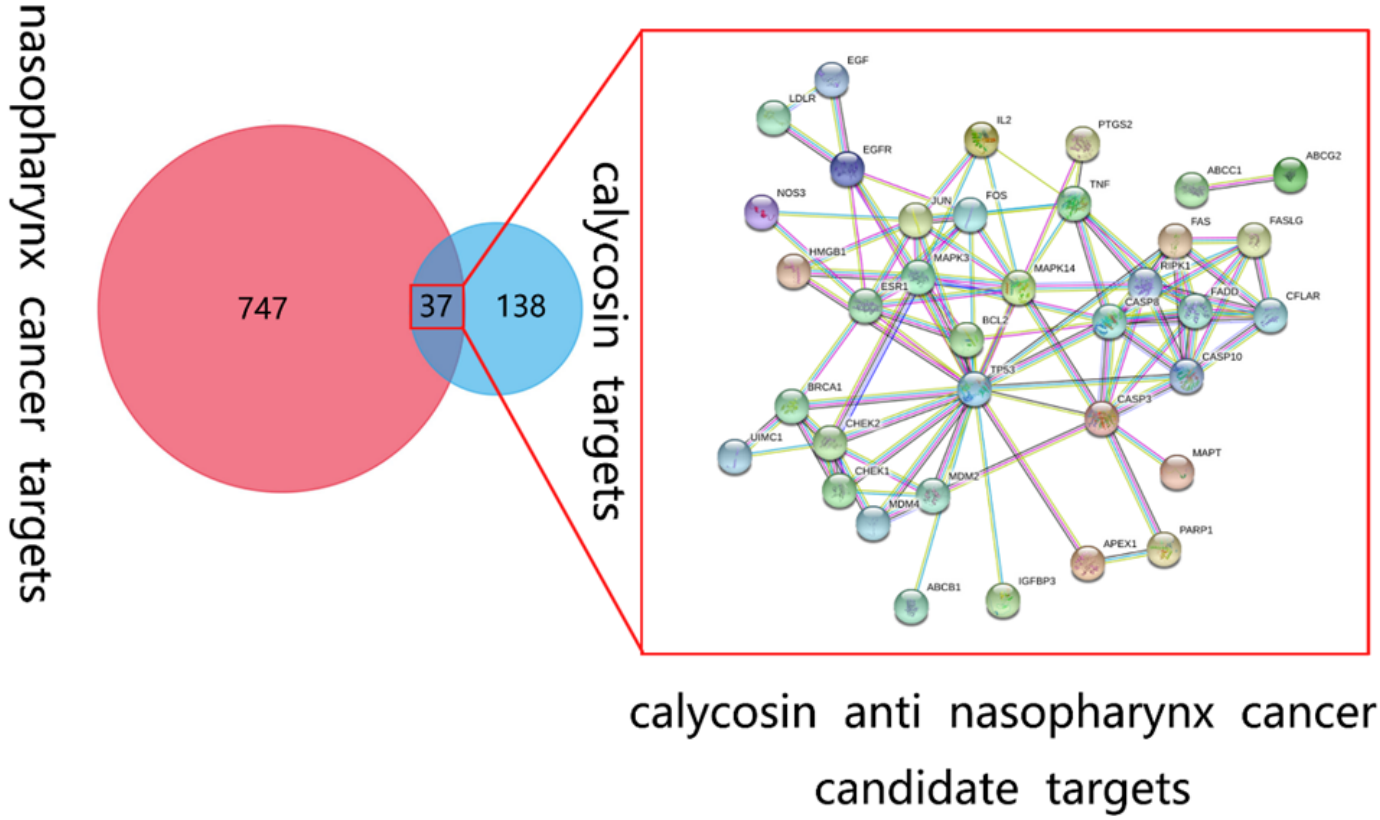

Figure 4

A mapped network from biotargets of calycosin against NPC.

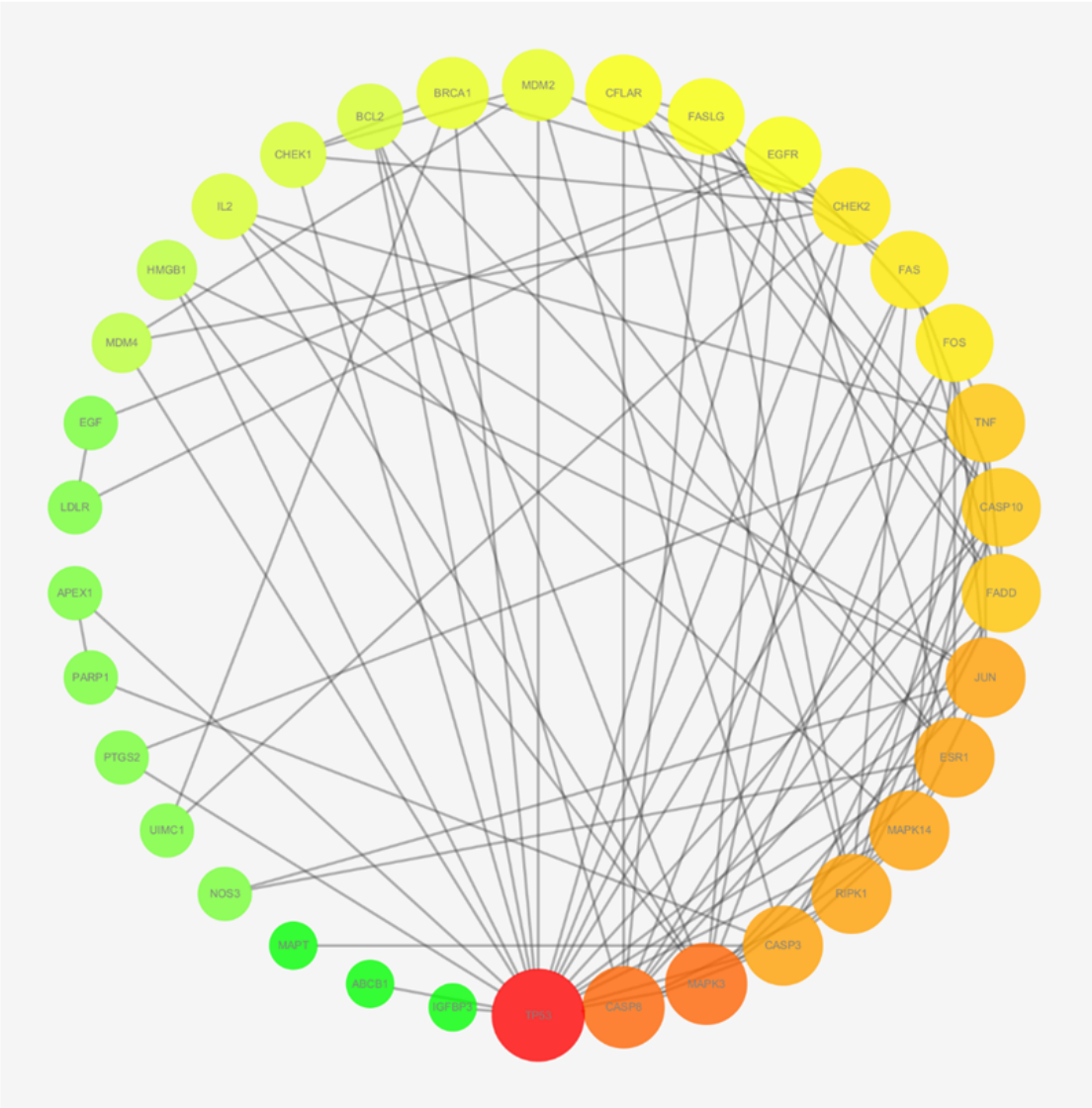

Figure 5

All candidate biotargets of calycosin and NPC.

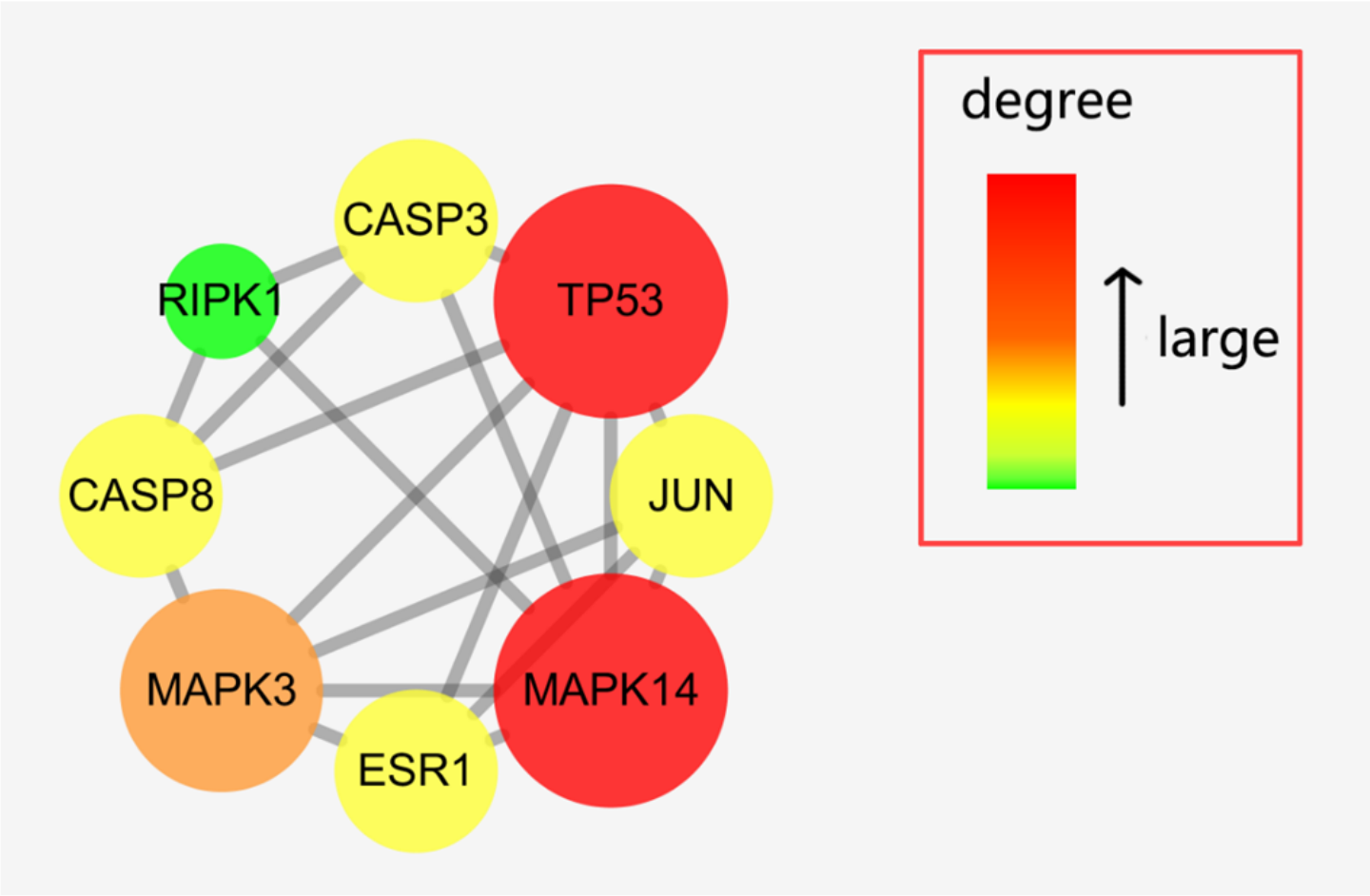

Figure 7

All hub biotargets.

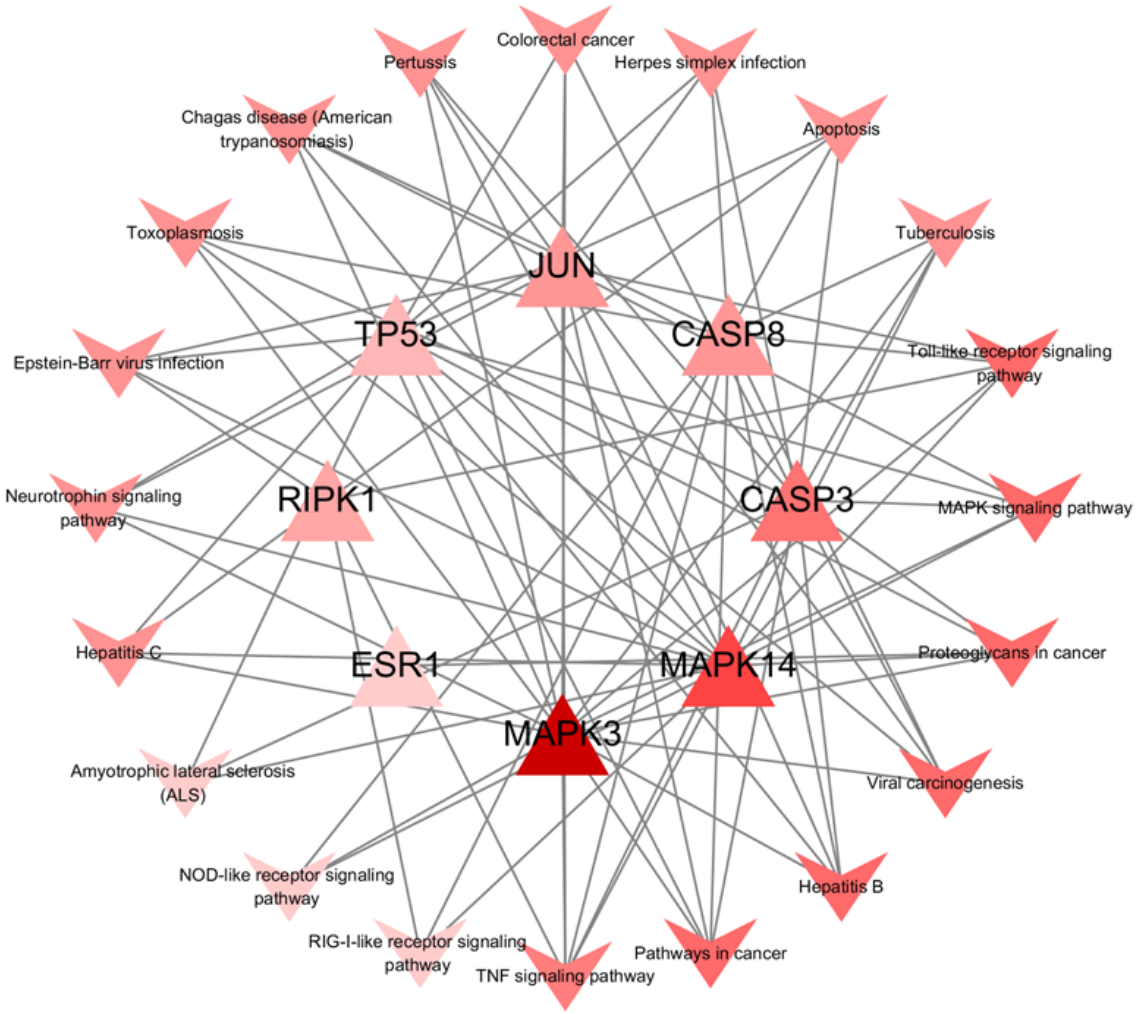

Figure 9

Cluster assays from PPI biotargets.

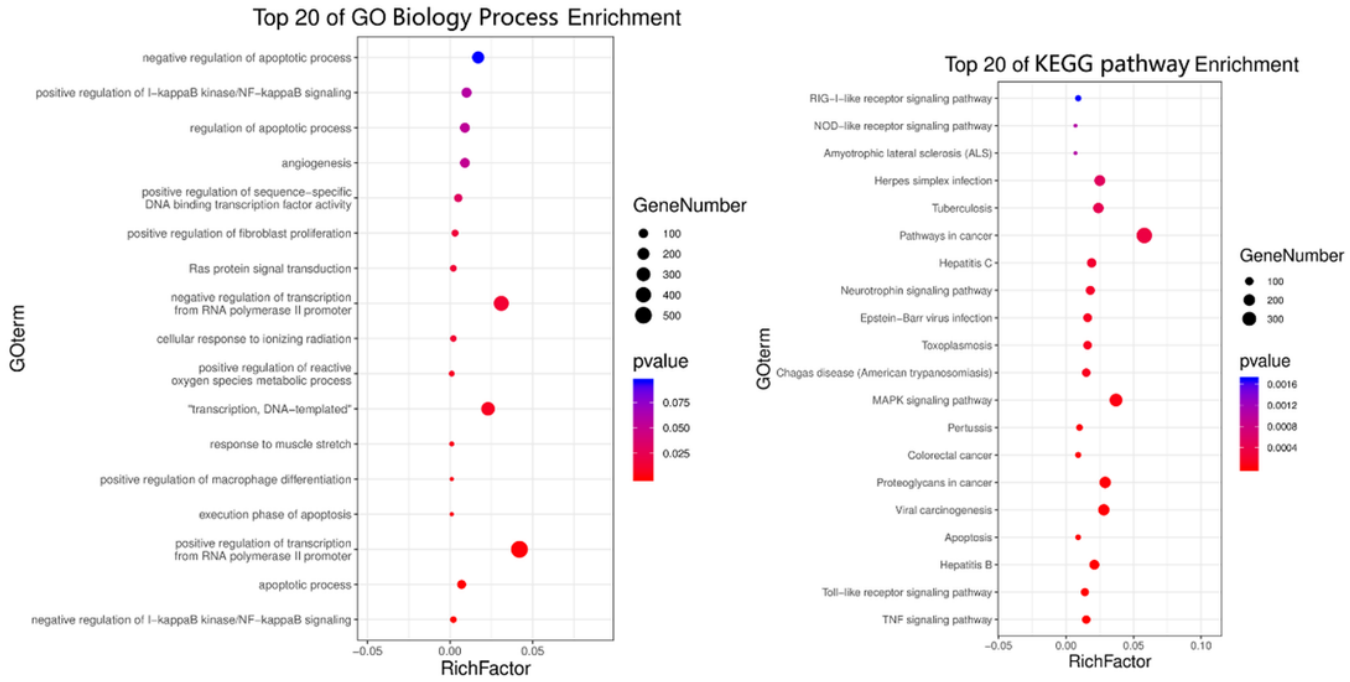

Figure 11

Enrichment analyses for top functional processes, molecular mechanism.

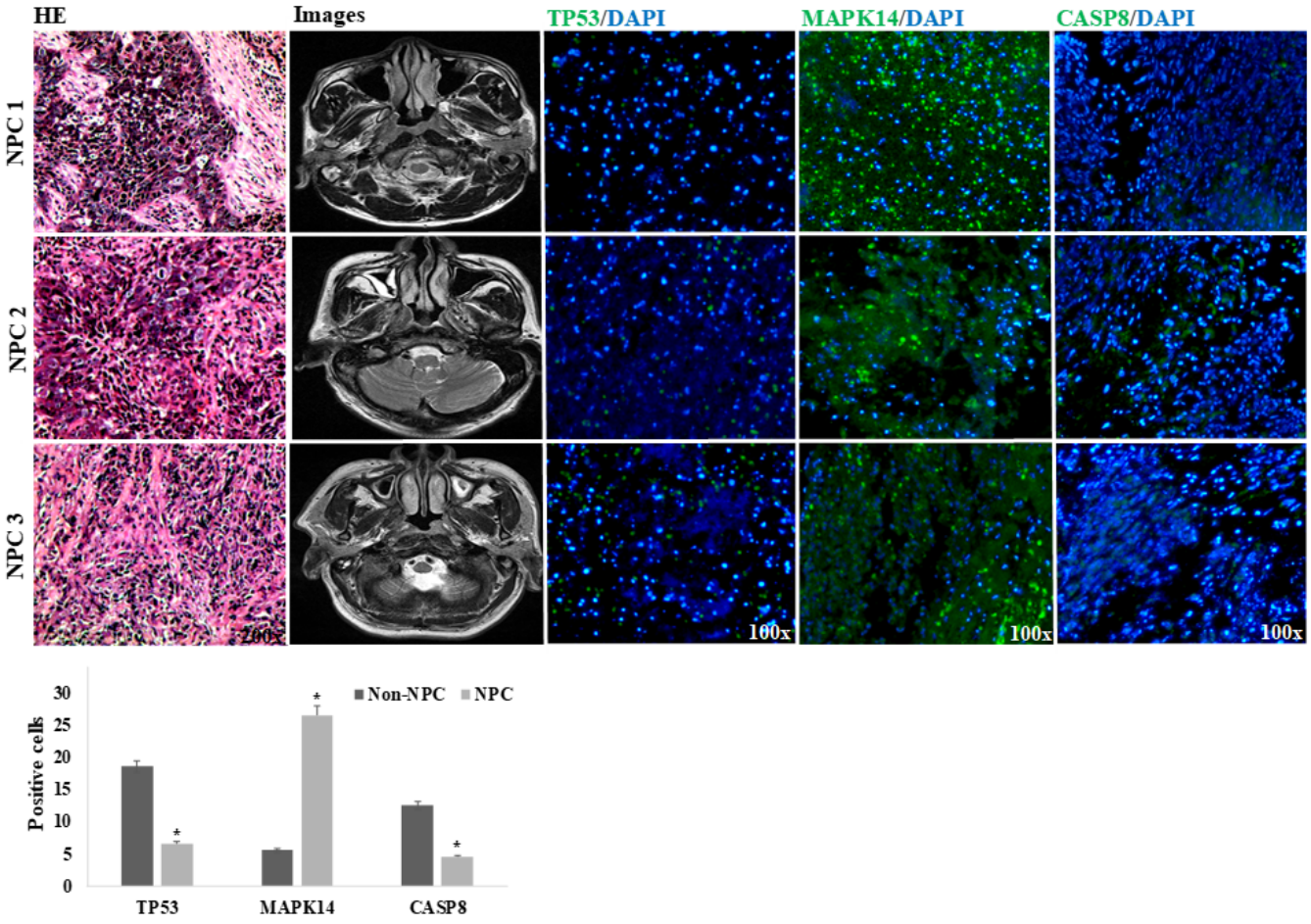

Figure 14

Clinical data and findings of patients with NPC.

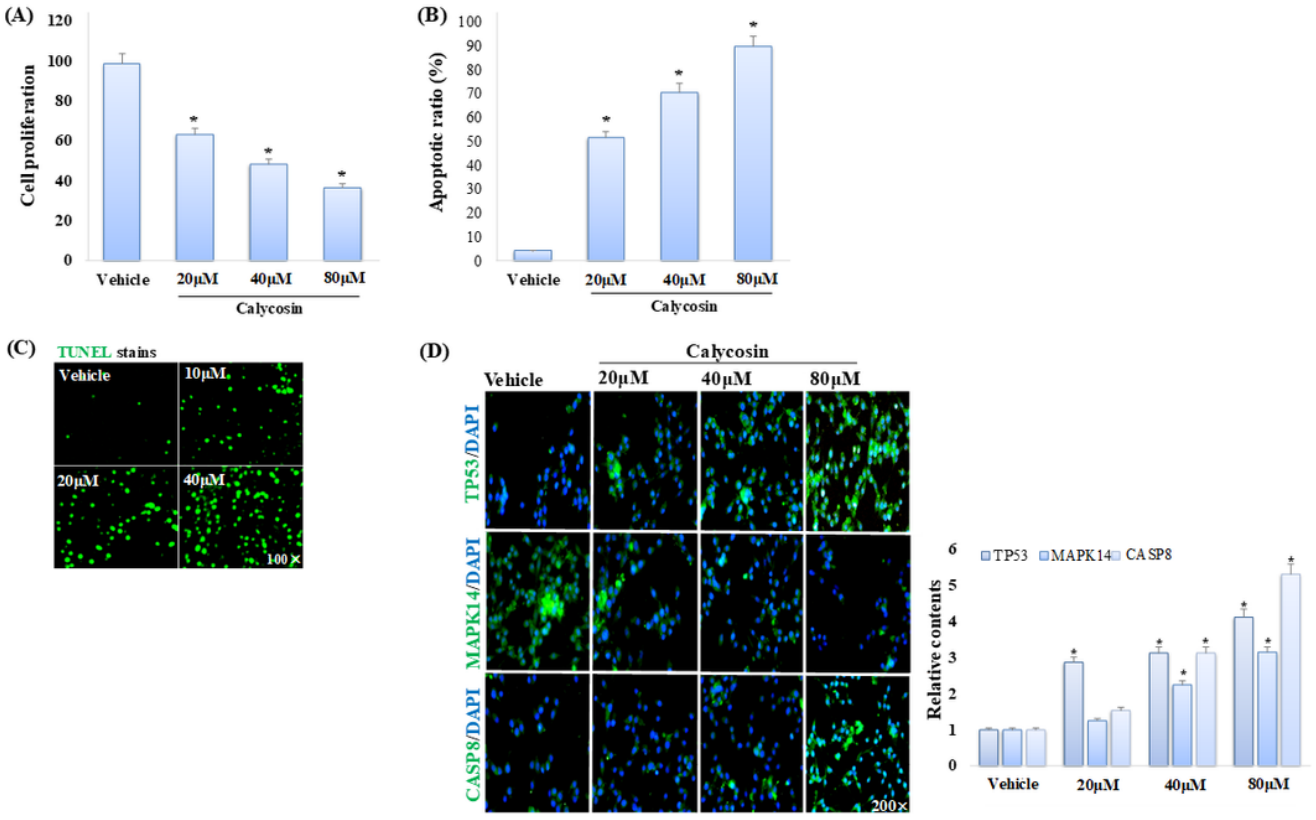

Figure 16

Anti-NPC pharmacological effect of calycosin in a cell line study.

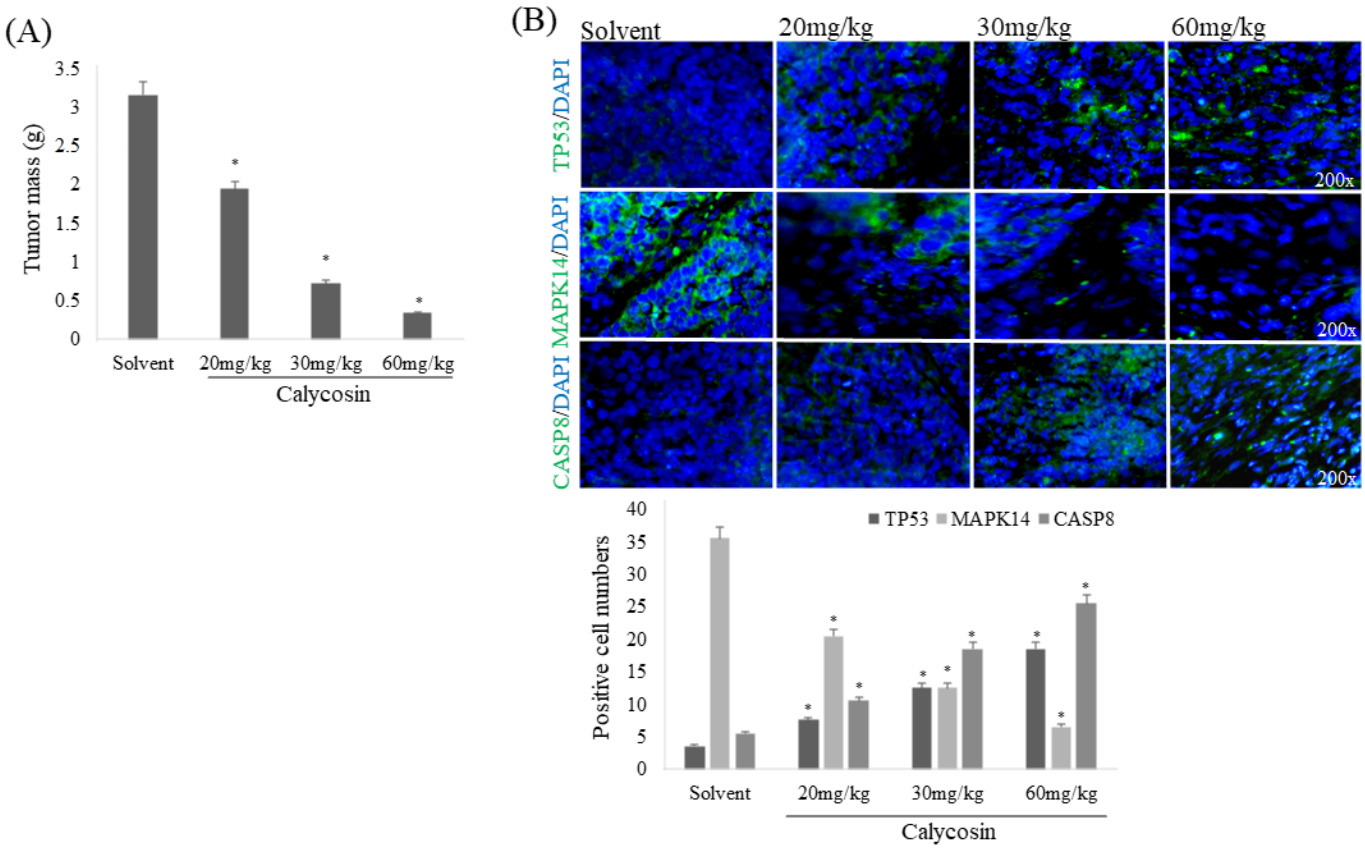

Figure 17

Anti-NPC pharmacological effect of calycosin in a tumor-bearing nude mice study.