Systematic and experimental approaches to indicate anti-tumor biotargets and molecular mechanisms of calycosin against osteosarcoma

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Research

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Abstract

Background

Calycosin is reported to pharmacologically combat cancer cells, however, the detailed anti-osteosarcoma (OS) mechanisms remain unclear. By using systemic method of network pharmacology, the present study purposed to reveal the anti-OS biotargets and molecular mechanisms played by calycosin. Moreover, human and animal experiments were used to verify the key biotargets of calycosin combating OS.

Results

In OS findings, the blood parameters showed increased tumor markers and OS sections resulted in negatively expressed proteins of tumor protein p53 (TP53), Caspase-3 (CASP3), X-linked Inhibitor of Apoptosis Protein (XIAP). To feature the pharmacological biotargets in a cell line study, calycosin-treated OS cells showed reduced cell proliferation, and promoted cell apoptosis. In TUNEL stains, calycosin resulted in elevated apoptotic cells. As showed in immunostaining, calycosin-treated OS cells suggested elevated TP53, CASP3, XIAP protein expressions.

Conclusions

Overall, our present human and cell culture data manifest all candidate and key biotargets, molecular functions and pathways of calycosin combating OS. Attractively, these top biotargets may be used for pharmacological hallmarks to combat OS.

Background

OS refers to a malignant bone cancer with a high recurrence and metastasis [1]. Clinically, the mortality rate is increased if unmanaged over time. However, the early symptoms and conditions of OS is pathologically inapparent. In consequence, the clinical diagnosis is proofed mainly with late stage in most OS patients when first inspection [2-3]. Meanwhile, the existing medical regimen for treating OS is limitedly presented, especially chemotherapy. More markedly, prescribing chemotherapeutics are associated with undesired harmful effects [4-5]. These reviewing evidences indicate that chasing another promising medicine against OS is became imperatively. In China, traditional herbs are rich in candidate compounds with potent pharmacological activities [6], such as artemisinin, berberine, curcumin. Interestingly, *Astragalus root* is used as a clinical injection for improving immunity and adjuvant treatment of cancer [7]. Calycosin, a natural ingredient extracted from *Astragalus root*, is pharmacologically managing cardiovascular diseases, cytoprotective, anti-aging, anti-oxidative effects [8]. Beneficially, calycosin is reportedly found with potent pharmacological activities against malignant cancer cells [9-10], including colorectal cancer, breast cancer, ovarian cancer, osteosarcoma. However, the detailed mechanism of calycosin against OS is not yet complete revealed. Intriguingly, a network pharmacology-based strategy is hopefully applied in systemic investigation of candidate biotargets and molecular mechanisms of herb-rich ingredient against clinical disorder [11-12]. In method, the system

pharmacological findings may further use to unveil all key targets, biological functions and signaling pathways of calycosin managing OS. Owe to limited data, our present study was designed to choose systemic pharmacology for revealing anti-OS effects exerted by calycosin before being created a hub map of calycosin managing OS and detailed therapeutic mechanisms. Additionally, some of integrative findings was to be certified with human data and cell line experiments. In brief, a hierarchical diagram using system pharmacology was demonstrated in current Figure 1.

Methods

Obtaining of candidate biotargets of calycosin managing OS

A group of emerging databases of SwissTargetPrediction, GeneCards, SuperPred was used to obtain all pharmacological hallmarks, while the OS-causing genes were accordingly collected through a disease-sorting tool of DisGeNET. And then all candidate targets of calycosin and OS were pooled together and mapped for identification of anti-OS biotargets by calycosin using bioinformatic setting [13].

Generation of a protein-protein interaction (PPI) map and selection of key targets

All available mapped targets were re-tested and used to create a functional correlative map of calycosin managing OS through a STRING platform. These data were further processed as a PPI map of calycosin managing OS via Cytoscape tool. By using a setup of network analyser with topological parameter and based on reference value data, all key targets of calycosin managing OS were collected respectively by using FunRich analysis tool [14].

Enriched determination of molecular functions and pathways of key targets

After FunRich test, all key targets of calycosin managing OS were entered a database of Functional Annotation Bioinformatics Microarray Analysis (DAVID) for creating available biological processes and signalling pathways of calycosin managing OS. According to the -Log p-value, a three-dimensional bar chart of biological processes and signalling pathways of calycosin managing OS was drew [15-16].

Human OS study

Firstly, ten hospitalized patients were registered from Department of Bone and Soft Tissue Surgery, Affiliated Tumor Hospital, Guangxi Medical University, and were diagnosed with OS through clinical images, histopathological examination, biochemical assays. The plasma samples of OS were used for biochemical test. And OS samples were prepared as 5μm sections for immunostaining. In a statement, the present study was approved by the Hospital Ethics Committee, and all human procedures were administered strictly based on terms of the Declaration of Helsinki [17-18].

A cell line study

A human malignant cell line of U2OS was commercially purchased from ATCC bioresource centre (USA). After arrival, the cells were kept in cell growing medium of Dulbecco's Modified Eagle Medium (DMEM) before dose-dependent treatments of calycosin (Sigma, USA, purity >98%; 0, 10, 20, 40μM) for around 48 hours. And then the cells were collected for a series of biological experiments [19-20].

Immunostaining tests

In methods, de-paraffin OS sections and paraformaldehyde-fixed U2OS cells were processed with 5% bovine serum albumin for blocking around 1 hour. After being washing with phosphate buffer saline/0.5% tween 20 for several times, the samples were incubated with different concentrations of primary antibodies of TP53 (1:100, Bioss, Beijing, China), CASP3 (1:100, Bioss, Beijing, China), XIAP (1:100, Bioss, Beijing, China) at 4°C overnight. And then the samples were further incubated with secondary antibody of IgG H&L (Alexa Fluor-488) (1:200, Abcam, USA). All cell nuclei were stained with a dye of 4,6-diamidino-2-phenylindole before positive cells were photographed by a fluorescence microscope (Olympus, Japan) [21-22].

Statistical analysis

Our current final data were expressed as the means ± standard deviations. Comparisons among different groups were presented by using a one-way analysis of variance matching with Tukey’s post hoc test. All statistics assays were implemented through a software of Statistical Product and Service Solutions 19.0 (Chicago, IL, USA). Significance was designed as a p-value less than 0.05 [23].

Results

Biological information of all candidate targets

Briefly, a group of 556 OS-causing genes were obtained accordingly. As a result, 101 predictive genes, 51 known genes, other 65 associated genes of calycosin were collected before final identification of 175 componential targets. After mapped, a functional PPI map of calycosin managing OS was created with all candidate targets (Figure 2-3).

Topology parameter analysis and identifying key targets

Network Analyzer was used to reveal topological parameters of target and functional protein interaction networks of calycosin managing OS. By calculating the degree of freedom, the average value was 37 and the maximum degree of freedom was 63. Therefore, the key target screening condition range was set to 37 to 63. As results, the top biotargets were screened and ordered as TP53, CASP3, XIAP, BCL2L11, ATM, CYCS, CASP8, BCL2L1 and CASP9 (Figure 4).

Cluster assay

As revealed in Figure 5, four cluster groups were created through a MCODE algorithm in setting of Cytoscape. In parallel, all combinatorial biotargets of calycosin managing OS were distinguished respectively.

Key molecular processes and biological pathways

By using FunRich tool, all key biotargets were used to construct the functional processes and signaling pathways through visualization assay. As results, the main biological processes of key biotargets were related to positive regulation of neuron apoptotic process, intrinsic apoptotic signalling pathway in response to DNA damage, apoptotic process, cellular process regulating host cell cycle in response to virus, regulation of apoptotic process, replicative senescence, execution phase of apoptosis, cellular response to gamma radiation, positive regulation of release of cytochrome c from mitochondria, neuron apoptotic process, extrinsic apoptotic signalling pathway in absence of ligand, cellular response to UV, cell cycle arrest, male gonad development, positive regulation of apoptotic process, in utero embryonic development, spermatogenesis. Furthermore, the dominating KEGG signalling pathways of key biotargets were linked with Apoptosis□Toxoplasmosis, p53 signalling pathway, Small cell lung cancer, Pathways in cancer, Amyotrophic lateral sclerosis (ALS) , Hepatitis B, Legionellosis, Colorectal cancer, Viral myocarditis, Huntington's disease, NF-kappa B signalling pathway, HTLV-I infection, Tuberculosis, Non-alcoholic fatty liver disease (NAFLD) , Herpes simplex infection, Alzheimer's disease, Pancreatic cancer, MicroRNAs in cancer, PI3K-Akt signalling pathway (Figure 6).

Human data of OS patients

Basically, these available OS patients presented an average age with 32.56±13.6 years and gender ratio of male:female=(5:1). As shown in Table 1, the increased trends of alkaline phosphatase, triglyceride, blood glucose, lactate dehydrogenase, high-sensitive C reactive protein, some immune cell counts in blood samples of OGS were observed. However, inapparent changes of trace element, hepatonephric functional enzymes, and cancer antigen contents were showed. In order to verify representatively some of key biotargets, OS samples were collected for immunostaining assays. As results, markedly reduced expressions of TP53, CASP3, XIAP in OS samples were detected, respectively (Figure 7).

Pharmacological action of calycosin managing OS

To further feature the beneficial effects of calycosin against OS, a cell culture study was conducted for pharmacological verification. Intriguingly, calycosin-dosed OS cells showed inhibited cell growth (P<0.05), and induced cell apoptosis (P<0.05) in a dose-dependent way. As shown in immunostaining assay, elevated expressions of TP53, CASP3, XIAP in calycosin-dosed OS cells were observed dose-dependently, and these positive cells of calycosin groups were less than those in non-dosed cells (P<0.05) (Figure 8).

Discussion

OS is a primary cancer with unmanaged cell proliferation and invasiveness prior to the OS cells are metastasis to other tissues [24]. By contrast, the current prescribing chemotherapeutic drugs against OS is absolutely insufficient for patients. Thereby, screening potential anti-OS ingredient is important task for all pharmacologists. Calycosin, a naturally-occurring phytoestrogen, plays the attractive pharmacological activities in pre-clinical studies. Notably, calycosin is effective anti-cancer component for managing malignancies, including OS. The main proposed mechanism is possibly through anti-proliferative action against OS cells [25-26]. However, the more detailed mechanisms of calycosin against OS is not yet totally known. Resultingly, this study was designed to use network pharmacology-based bioinformatic strategy to highlight all pharmacological hallmarks and mechanisms of calycosin managing OS. As revealed in current bioinformatic findings, the main functional processes of calycosin against OS were implicated in modulating positive regulation of neuron apoptotic process, intrinsic apoptotic signalling pathway in response to DNA damage, apoptotic process, cellular process regulating host cell cycle in response to virus, regulation of apoptotic process, replicative senescence, execution phase of apoptosis, cellular response to gamma radiation, positive regulation of release of cytochrome c from mitochondria, neuron apoptotic process, extrinsic apoptotic signalling pathway in absence of ligand, cellular response to UV, cell cycle arrest, male gonad development, positive regulation of apoptotic process, in utero embryonic development, spermatogenesis. Functionally, existing biological processes were inextricably linked to the development of nerves, apoptosis, indicating that the calycosin may exert the anti-osteosarcoma effects by regulating these key biological processes. Moreover, the top crucial signalling pathways of calycosin managing OS were completely revealed. In addition, After obtaining all candidate biotargets of calycosin and OS, the key biotargets were highlighted and top 3 biotargets would be certified using OS and cell line 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 [27]. Pathologically, abnormal mutation of TP53 is found with many cancer human and cell samples, marked by malignant proliferation, uncontrolled growth, and strong invasiveness [28]. CASP3, a cell-death protease, plays a key role in apoptosis for cell death. In cancer cells, CASP3 is suppressed functionally for uncontrolled proliferation, invasiveness and metastasis [29-30]. XIAP, another apoptotic suppressor protein, is required for the anti-apoptotic function. Defects in XIAP are one of leading causes of malignant lymphoma and other cancers [31-32]. Accordingly, these predictive biotargets will be certified by experiments. As results, the current OS samples showed markedly up-regulated expressions of TP53, XIAP, reduced CASP3 protein. And the medical images, and blood cancer markers were checked respectively. In further study *in vitro*, a human cell line with calycosin treatments was conducted. The present results indicated that calycosin-treated OS cells with reduced cell growth, increased cell apoptosis and cell toxicity. These findings suggested anti-proliferative activity was responsible for underlying pharmacological mechanism of calycosin managing OS. In addition, further molecular mechanism needs to be discussed. In immunoassays, the pharmacological findings following calycosin treatments for SW620 cells highlighted that inducing TP53 to inhibit DNA repair, cell growth, promoting XIAP to block cell proliferation, decrease cell death, and up-regulating CASP3 to induce cell apoptosis. More interestingly, all these human and cell line data were

matched for top functional processes and molecular pathways of calycosin managing OS in bioinformatic assays.

Conclusion

To sum up, our network pharmacology-based and experimentative data unveil all candidate, key targets, functional processes and pharmacological mechanisms of calycosin managing OS. Moreover, some representative findings were certified with human and cell culture studies.

Abbreviations

OS, osteosarcoma; TP53, tumor protein p53; CASP3, Caspase-3, XIAP, X-linked Inhibitor of Apoptosis Protein; TUNEL, Terminal Deoxynucleotidyl Transferase; PPI, protein-protein interaction; DAVID, Functional Annotation Bioinformatics Microarray Analysis; DMEM, Dulbecco's Modified Eagle Medium.

Declarations

Ethical Approval and Consent to participate

Not applicable.

Consent for publication

Not applicable.

Availability of supporting data

Not applicable.

Competing interests

None.

Funding

Not applicable.

Authors' contributions

Zhenjie Wu, Zhenchao Yuan for Research Project with Conception, Organization, Execution; Jiachang Tan, Xiong Qin, Bin Liu, Hao Mo for Statistical Analysis with Design, Execution, Review and Critique

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Authors' information

Not applicable.

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Table

Table 1 Medical indicators of patients with **osteosarcoma (n=10)**

Indicators	Mean ± SD	Clinical range
WBC(10^9/L)	6.77±1.37	4-10
NEUT%	58.46±8.94	0.37-0.72
EO%	2.71±2.51	0-0.06
BASO%	0.75±0.71	0-0.01
LYMPH(%)	31.1±9.63	0.2-0.5
MONO(%)	6.94±1.44	0-0.14
RBC(1012/L)	121.9±16.33	3-5.5
HGB(g/L)	103.67±15.18	110-150
PCT%	0.31±0.09	0.11-0.28
MCV(fL)	84.9±5.84	82-94
MCH(pg)	27.7±2.66	26-32
MCHC(g/L)	325.9±41.27	320-360
RDW-SD(FL)	41.27±6.81	37-54
MPV(fL)	8.58±0.8	6--15
PDW(fL)	15.65±0.41	10--15
RDW-CV(%)	14.01±2.82	0.11-0.16
LYMPH(10^9/L)	2.1±0.67	1-3.7
MONO(10^9)	0.46±0.14	0—0.7
NEUT(10^9/L)	3.98±1.08	1.5-7
EO(10^9/L)	0.17±0.13	0-0.4
BASO(10^9/L)	0.05±0.03	0-0.1
P-LCR(%)	16.89±5.19	0.13-0.43
HCT(%)	37.43±4.51	0.37-0.47
PLT(10^9/L)	366.8±126.06	100-300

TBIL(μmol/l)	8.53±2.94	0.001-21
DBIL(μmol/l)	3.19±0.97	0.001-6
IBIL(μmol/l)	5.34±2.17	1.71-15
ALT (U/l)	15.10±8.82	0.001-38
AST (U/l)	36.30±40.55	0.001-40
ALP (U/l)	284.40±227.92	25-90
GRF (U/l)	84.82±41.3	74-150
TBA(μmol/l)	3.82±1.82	0.001-20
ADA (U/l)	13.87±8.44	0-25
TG (g/l)	3.65±0.18	0.23-1.71
ALB (g/l)	40.48±3.86	35-55
GLO(g/l)	26.61±3.73	15-33
CHOL(ng/mL)	4.32±0.74	3.6-6
GLU(mmol/L)	4.31±0.42	3.9-6.1
Mg(mmol/l/l)	0.93±0.19	0.66-1.07
BUN (mmol/L)	4.78±2.08	3.2-7.5
CR(μmol/l)	60.5±26.42	44-115
UA (μmol/l)	320.56±144.3	110-420
CHOL(mmol/L)	4.32±0.74	3.12-6.24
A/G(%L)	1.51±0.20	1.3-2.7
HDL-C(mmol/L)	1.22±0.3	0.7-2
LDL-C(mmol/L)	915.33±1758.05	1.76-3.49
Apo A(g/l)	1.05±0.21	1-1.6
Apo B(g/l)	0.72±0.12	0.6-1.1
LDH(U/l)	915.33±1758.05	114-240
CHE(U/L)	6571.70±1615.81	4000-13200
K(mmol/l)	4.41±0.48	3.2-5.2
Na(mmol/l)	141.50±1.58	135-145
Cl(mmol/l)	103.50±3.03	98-110
Ca(mmol/l)	2.54±0.17	2.1-2.55
P(mmol/l)	1.35±0.17	0.87-1.6
TP(g/l)	58.5±2.26	60-83
PA(mg/l)	217.2±76.32	180-390
r-GT(U/L)	18.7±5.29	0.001-45

Figures

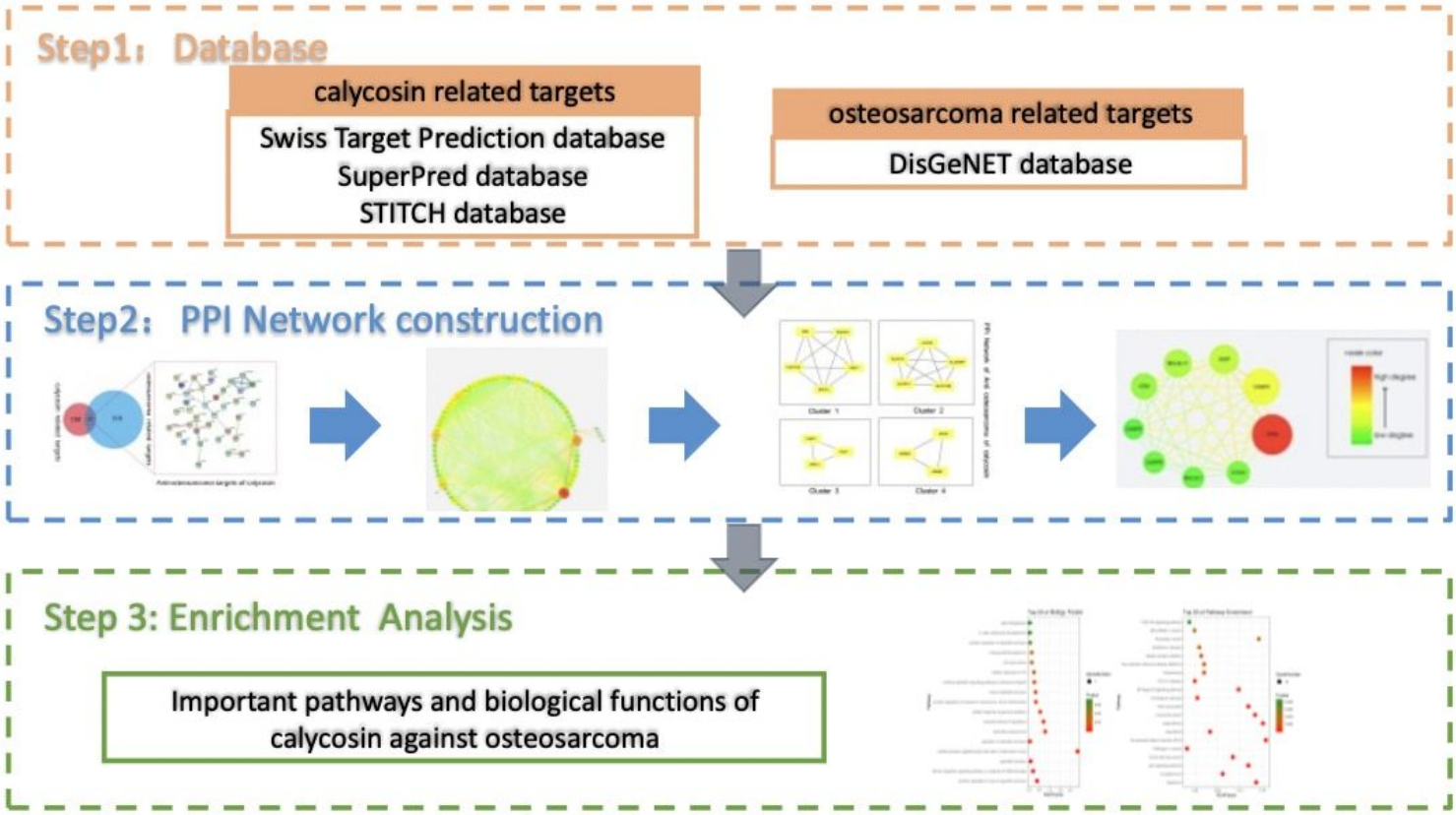

Figure 2

A network pharmacology-based diagram for the present manuscript.

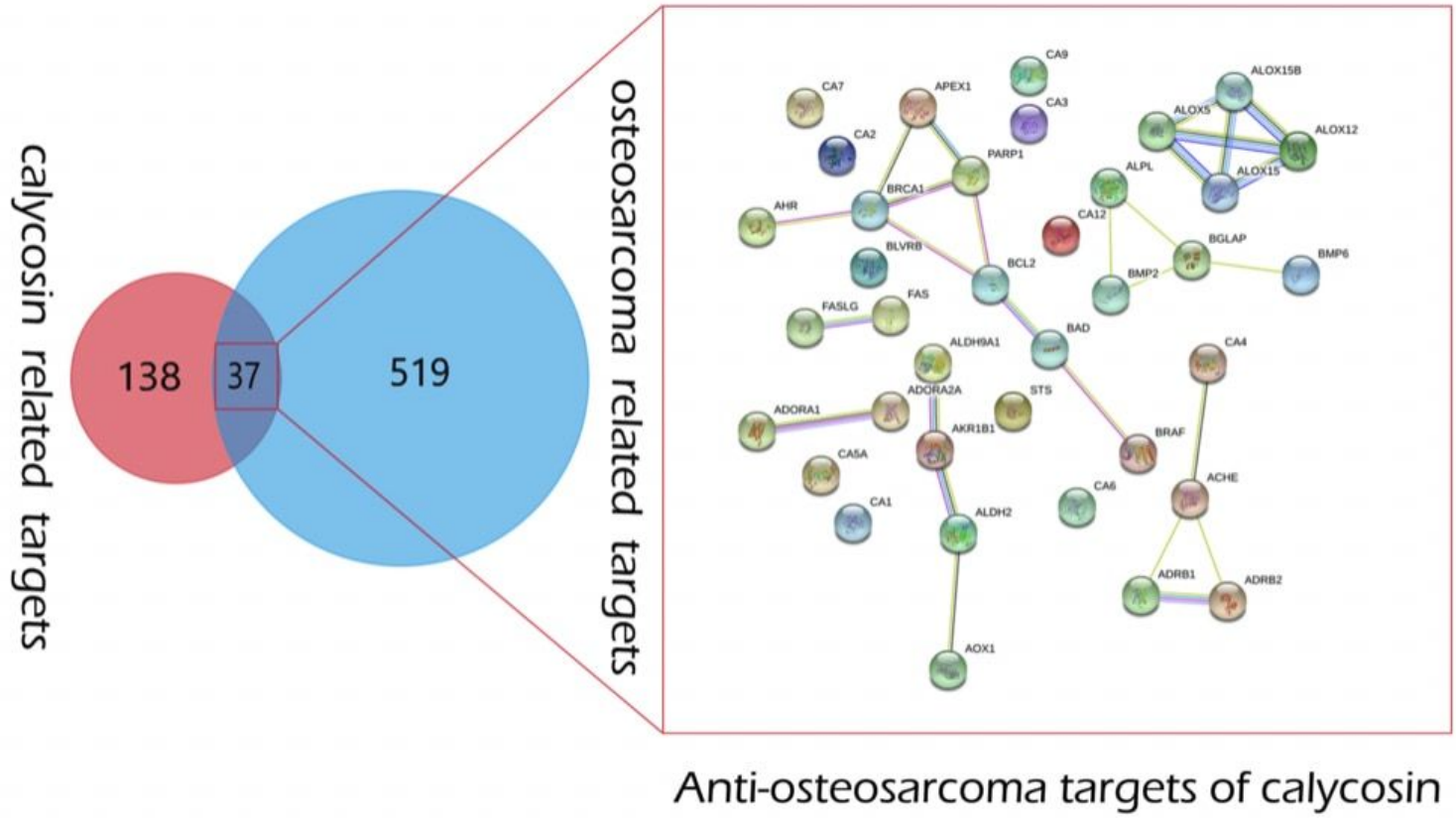

Figure 3

A mapped network from biotargets of calycosin managing OS.

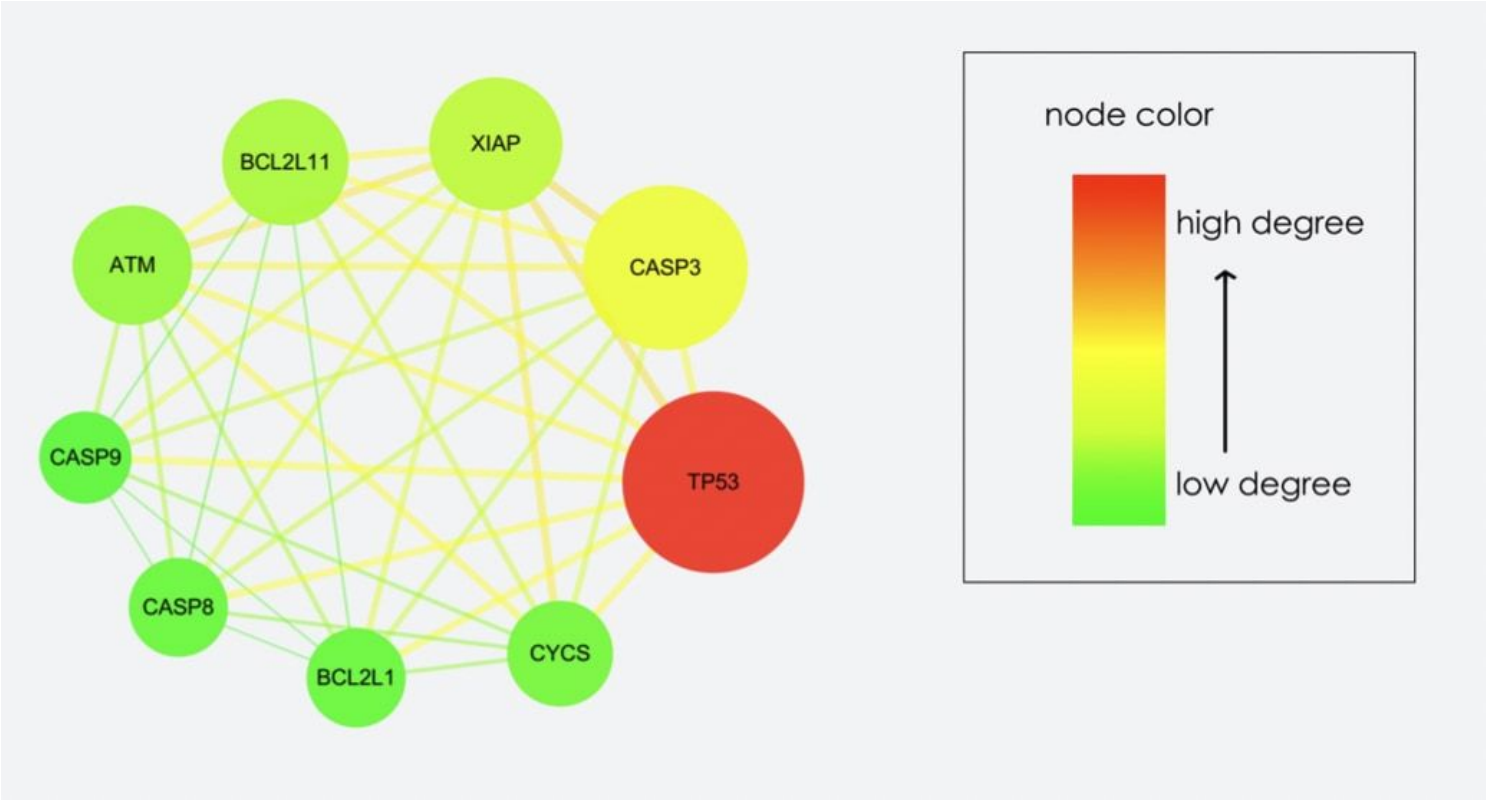

Figure 5

All candidate biotargets of calycosin and OS.

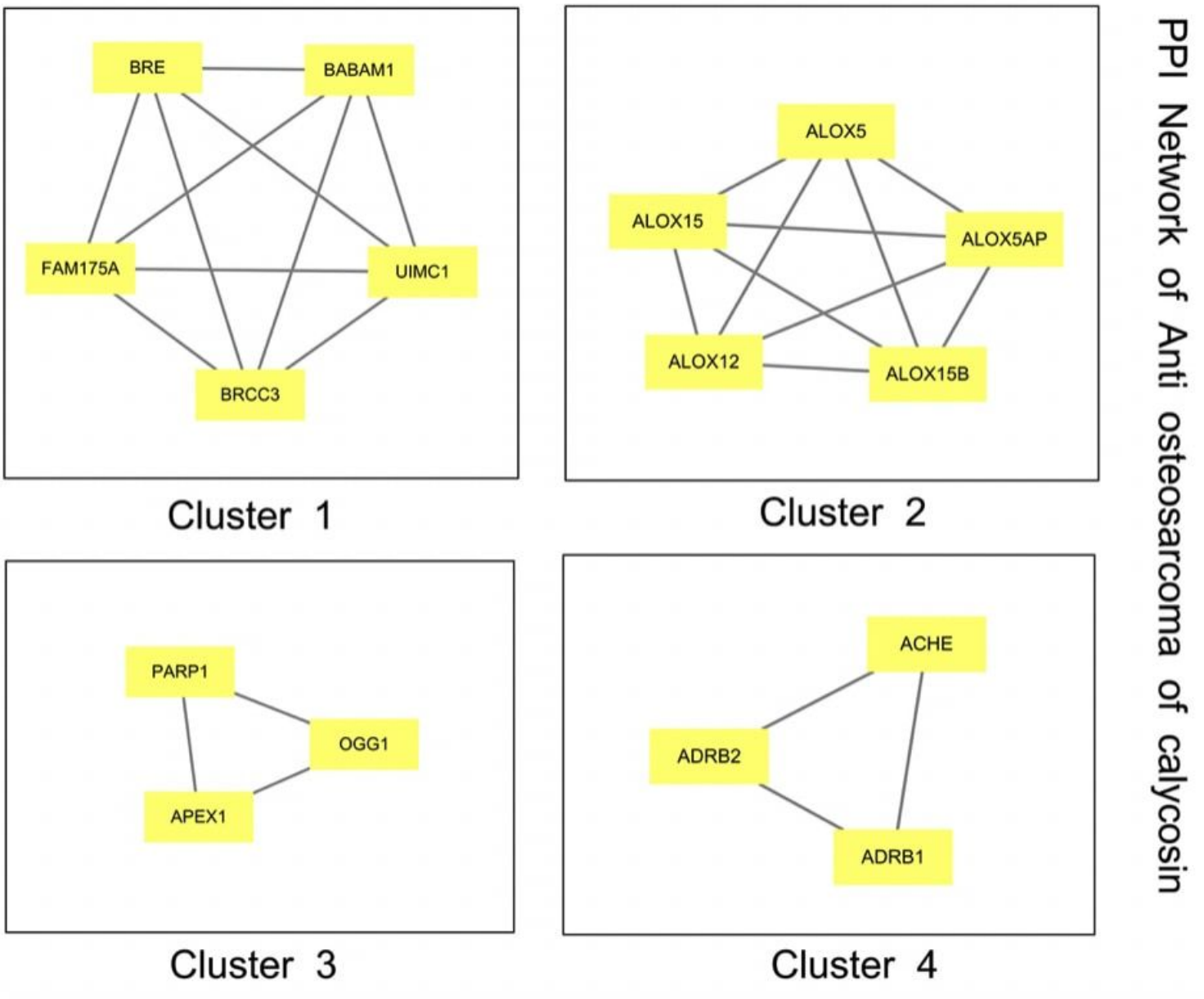

Figure 7

All hub biotargets.

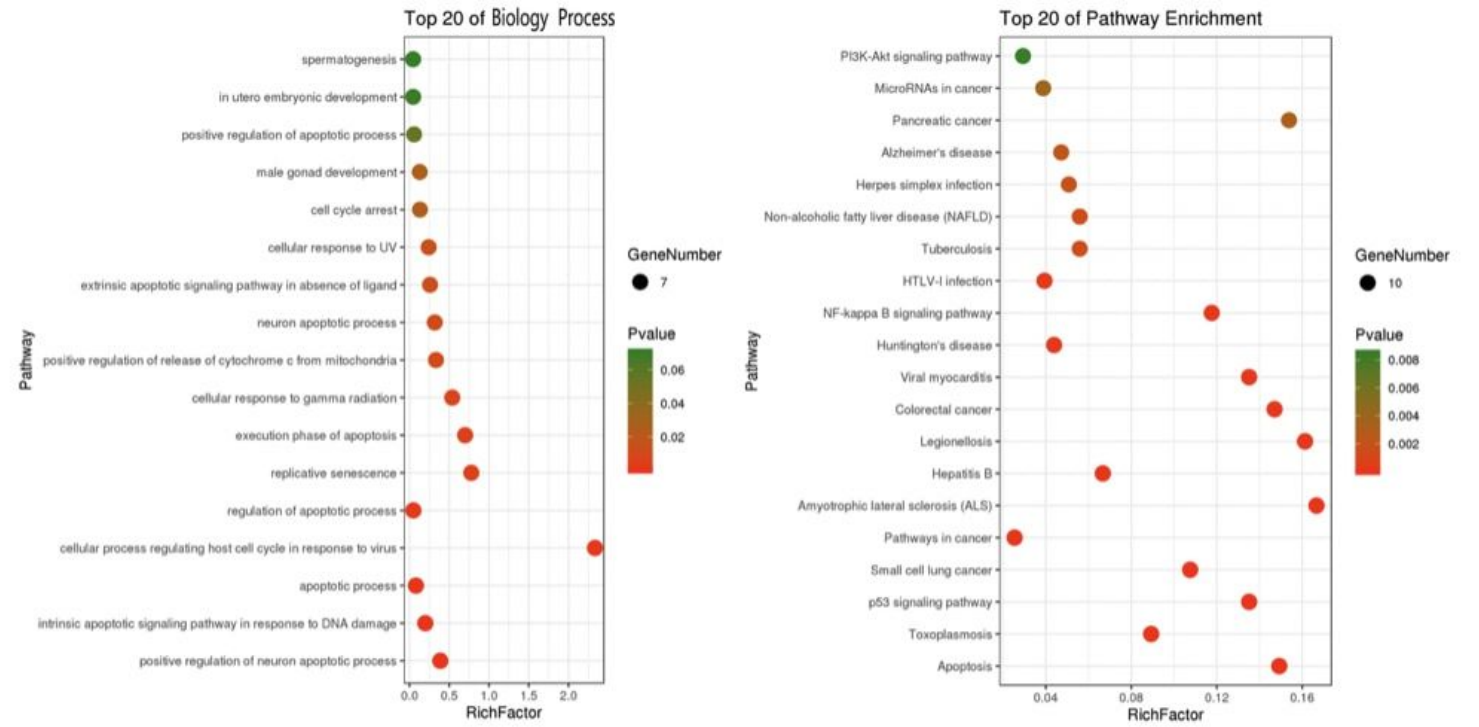

Figure 10

Cluster assays from PPI biotargets.

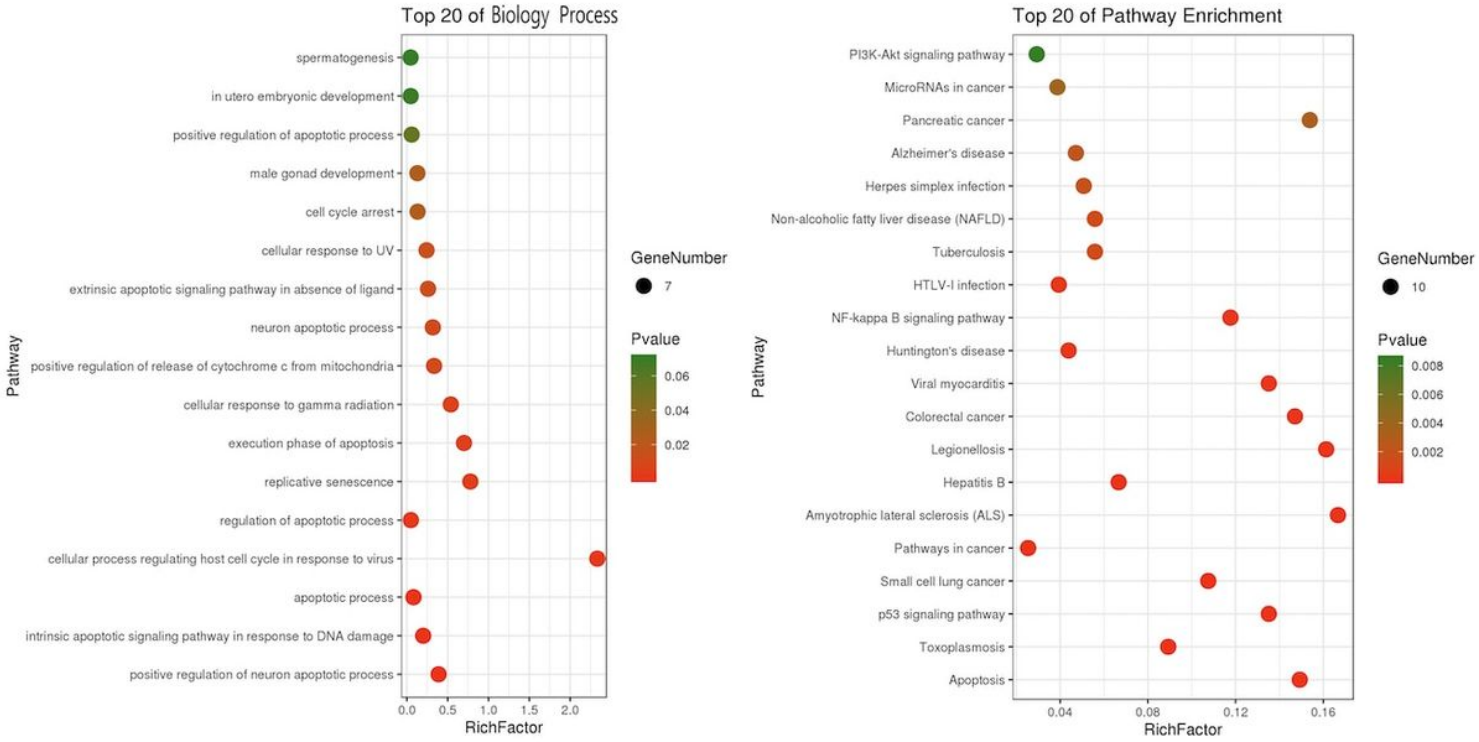

Figure 12

Enrichment analyses for top functional processes, molecular mechanism,

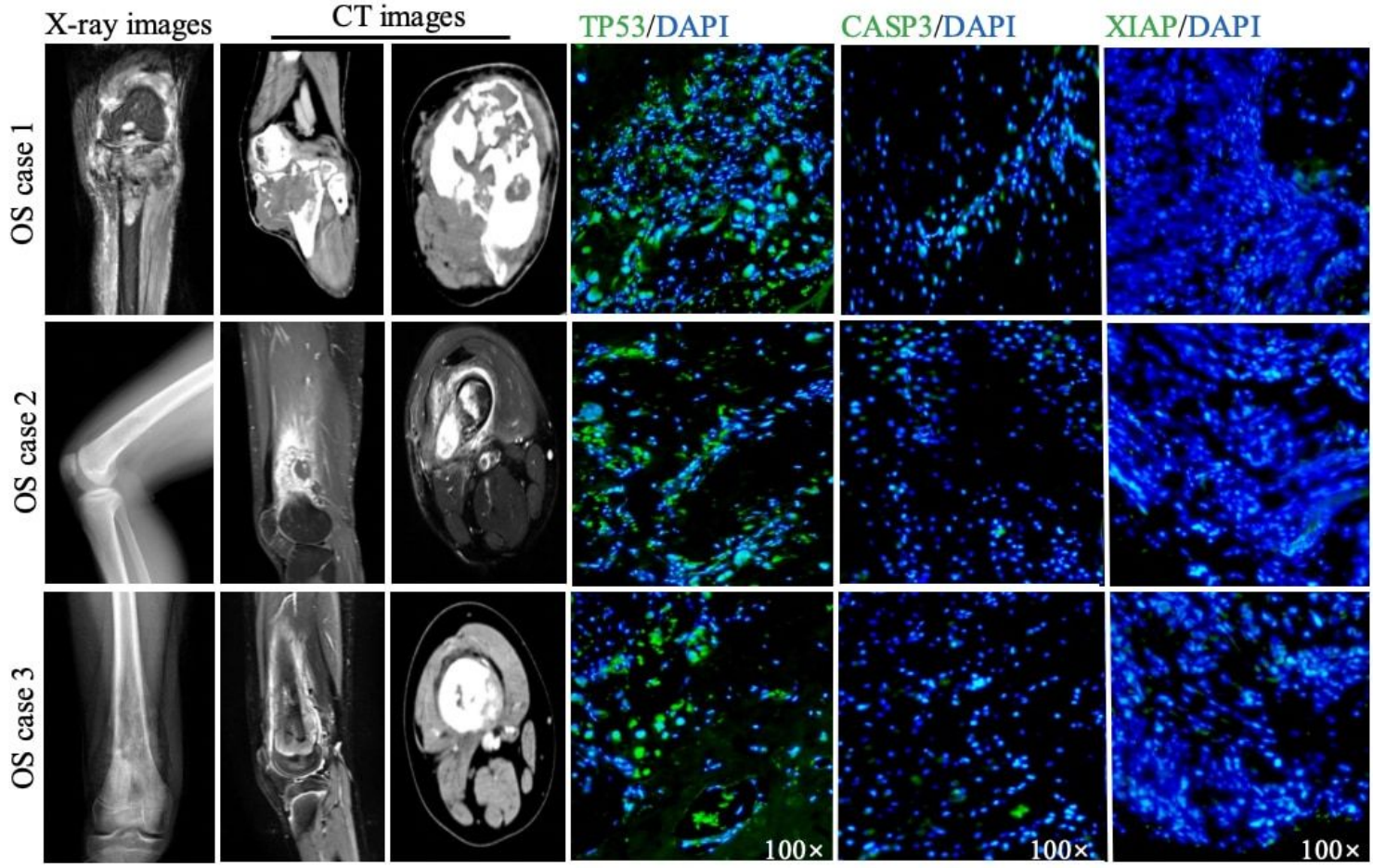

Figure 14

Clinical data and findings of patients with OS

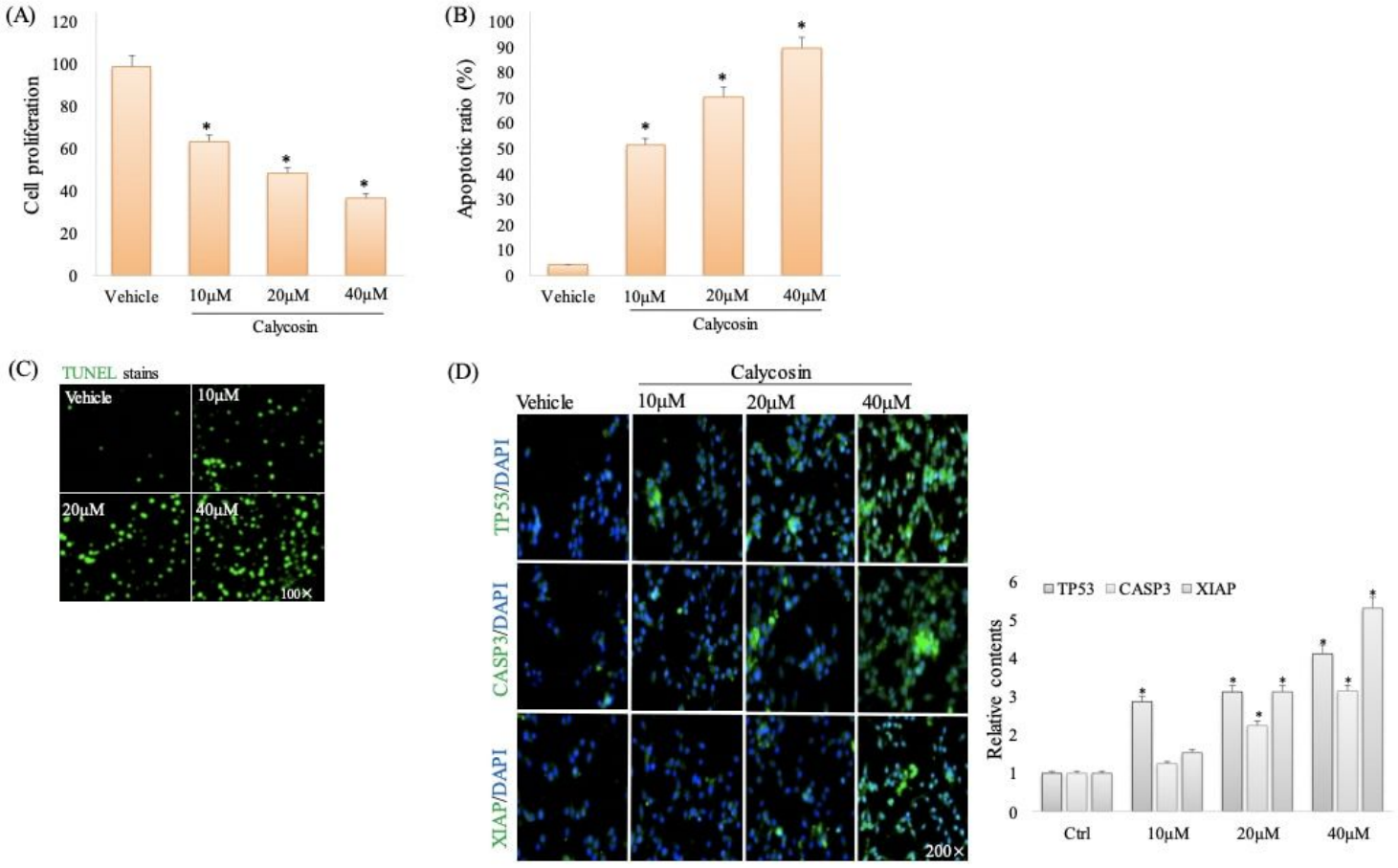

Figure 16

Anti-OS pharmacological action of calycosin in a cell line study